(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 November 2001 (15.11.2001)

PCT

(10) International Publication Number
WO 01/84933 A1

(51) International Patent Classification[7]: A01N 63/00, A61K 48/00, C12N 5/00, 5/02, 5/08, 15/00, 15/63, C12Q 1/00

(21) International Application Number: PCT/US01/15290

(22) International Filing Date: 11 May 2001 (11.05.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/569,259 11 May 2000 (11.05.2000) US

(71) Applicant: **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK** [US/US]; West 116th Street and Broadway, New York, NY 10027 (US).

(72) Inventors: **JESSELL, Thomas, M.**; 4621 Waldo Avenue, Bronx, NY 10471 (US). **BRISCOE, James**; 40 Parfrey Street, Hammersmith, London W69EN (GB). **ERICSON, Johan**; Ridderviksv, 147, S-165 72 Hasselby (SE).

(74) Agent: **WHITE, John, P.**; Cooper & Dunham LLP, 1185 Avenue of Americas, New York, NY 10036 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/84933 A1

(54) Title: A HOMEODOMAIN PROTEIN CODE SPECIFYING PROGENITOR CELL IDENTITY AND NEURONAL FATE IN THE VENTRAL NEURAL TUBE

(57) Abstract: Provided are genetically engineered cells comprising a neural stem cell and retroviral expression system in the neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein; which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein; and which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein. Also provided are methods of generating such genetically engineered motor neurons, V2 neurons, and V3 neurons. Also provided are methods of treating subjects having a motor neuron injury or a motor neuron disease comprising implanting in injured/diseased neural tissue of the subject any of the provided genetically engineered cells, administering to such neural tissue retroviral expression systems which are capable of expressing the appropriate homeodomain protein(s), or transfecting neural stem cells with a retroviral vector, which is capable of expressing the required homeodomain transcription factor protein(s). Provided is a method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell.

A HOMEODOMAIN PROTEIN CODE SPECIFYING PROGENITOR CELL IDENTITY AND NEURONAL FATE IN THE VENTRAL NEURAL TUBE

The invention disclosed was herein made in the course of work under NIH Grant No. RO1 NF33245-07. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims.

BACKGROUND OF THE INVENTION

In many developing tissues, the generation of distinct cell types is initiated by the action of extracellular signals provided by local organizing centers. Certain signals have the additional feature of directing distinct cell fates at different threshold concentrations, and thus function as morphogens (Wolpert, 1969). In *Drosophila*, the patterning of embryonic segments and imaginal discs involves the graded signaling activities of the Hedgehog, Wingless and TGFβ-related proteins (Lawrence and Struhl, 1996). In vertebrate embryos the specification of mesodermal cell types has similarly been suggested to depend on the graded signaling activity of members of the TGFβ family (Smith, 1995; McDowell and

Gurdon, 1999). The generation of cell pattern through morphogen signaling demands an effective means of converting graded extracellular activities into all-or-none distinctions in cell fate. But the mechanisms used to achieve such conversions have been poorly defined, particularly in vertebrate tissues.

In the developing vertebrate nervous system, Sonic hedgehog (Shh) appears to function as a gradient signal. The secretion of Shh by the notochord and floor plate controls the specification of ventral cell types (Marti et al., 1995; Roelink et al., 1995; Chiang et al., 1996; Ericson et al., 1996). Five distinct classes of ventral neurons can be generated in vitro in response to progressive two-to-three fold changes in extracellular Shh concentration (Ericson et al., 1997a, b). Moreover, the position at which each of these neuronal classes is generated in vivo is predicted by the concentration of Shh required for their induction in vitro: neurons generated in progressively more ventral regions of the neural tube require correspondingly higher concentrations of Shh for their induction (Ericson et al., 1997a). These observations have led to the view that the position that ventral progenitor cells occupy within a ventral-to-dorsal gradient of extracellular Shh activity directs their differentiation into specific neuronal subtypes (Ericson et al., 1997b).

In turn, these findings have focused attention on the steps by which graded Shh signaling directs the diversification of neural progenitor cells. Several

homeodomain proteins, Pax7, Pax3, Pax6, Dbx1, Dbx2 and Nkx2.2, are expressed by ventral progenitor cells and their expression is regulated by Shh signaling (Goulding et al., 1993; Ericson et al., 1996; Ericson et al., 1997a; Briscoe et al., 1999; Pierani et al., 1999). Moreover, the pattern of generation of certain ventral neuronal subtypes is perturbed in mice carrying mutations in these *Pax* genes and in the *Nkx2.2* gene (Ericson et al., 1997a; Mansouri and Gruss, 1998; (Briscoe et al., 1999), supporting the view that homeodomain proteins expressed by ventral progenitor cells regulate neuronal subtype identity. However, two important aspects of the link between Shh signaling and neuronal identity remain obscure. First, it is unclear how the presumed extracellular gradient of Shh activity results in stable and sharply delineated domains of homeodomain protein expression within ventral progenitor cells. Second, the spatial information provided by the homeodomain proteins characterized to date is insufficient to explain the diversity of neuronal subtypes generated at different dorsoventral positions.

In the first series of experiments these two issues are addressed. It is show first that the homeodomain proteins Nkx6.1 and Irx3 are expressed by progenitor cells in discrete domains of the ventral neural tube and are regulated by graded Shh signaling. The differential expression of five class I (Shh-repressed) proteins, Pax7, Irx3, Dbx1, Dbx2 and Pax6, and two class II (Shh-induced) proteins, Nkx6.1 and Nkx2.2, subdivides the ventral neural tube into five cardinal progenitor

domains. Misexpression of individual proteins in the neural tube in vivo in these experiments provides evidence that cross-repressive interactions between class I and class II proteins establish individual progenitor domains and maintain their sharp boundaries, suggesting a mechanism by which graded Shh signals are converted into all-or-none distinctions in progenitor cell identity. In addition, the experiments show that the spatial patterns of expression of Nkx6.1, Irx3 and Nkx2.2 are sufficient to direct both the position and fate of three neuronal subtypes generated in ventral third of the neural tube. These findings suggest a model of ventral neuronal patterning that may provide insight into how extracellular signals are interpreted during the patterning of other vertebrate tissues.

Distinct classes of neurons are generated at defined positions in the ventral neural tube in response to a gradient of Sonic Hedgehog (Shh) activity. A set of homeodomain transcription factors expressed by neural progenitors act as intermediaries in Shh-dependent neural patterning. These homeodomain factors fall into two classes: class I proteins are repressed by Shh and class II proteins require Shh signaling for their expression. The profile of class I and class II protein expression defines five progenitor domains, each of which generates a distinct class of post-mitotic neurons. Cross-repressive interactions between class I and class II proteins appear to refine and maintain these progenitor domains. The combinatorial expression of three of these proteins - Nkx6.1, Nkx2.2 and Irx3 - specifies the

identity of three classes of neurons generated in the ventral third of the neural tube.

Sonic hedgehog (Shh) signaling has a critical role in the control of neuronal fate in the ventral half of the vertebrate central nervous system (CNS). The genetic programs activated in Shh-responsive progenitor cells, however, remain poorly defined. To test whether members of the Nkx class of homeobox genes have a prominent role in the specification of ventral cell types the second series of experiments examined patterns of neurogenesis in mice carrying a targeted mutation in the Nkx class homeobox gene *Nkx6.1*. In *Nkx6.1* mutants there is a dorsal-to-ventral switch in the identity of progenitor cells and in the fate of post-mitotic neurons. At many axial levels there is a complete block in the generation of V2 interneurons and motor neurons and a compensatory ventral expansion in the domain of generation of V1 neurons. These studies support the idea that an *Nkx* gene code controls regional pattern and neuronal fate in the ventral region of the mammalian CNS.

SUMMARY OF THE INVENTION

This invention provides a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein.

This invention provides a method of generating a genetically engineered motor neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein so as to thereby generate the genetically engineered motor neuron.

This invention also provides a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein.

This invention further provides a method of generating a genetically engineered V2 neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein, under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein so as to thereby generate the genetically engineered V2 neuron.

This invention provides a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx.2.2 protein or homeodomain transcription factor Nkx2.9 protein.

This invention also provides a method of generating a genetically engineered V3 neuron which is capable of expressing homeodomain transcription factor Nkx.2.2 protein or homeodomain transcription factor Nkx2.9 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx.2.2 protein or homeodomain transcription factor

Nkx2.9 protein, under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx.2.2 protein or homeodomain transcription factor Nkx2.9 protein so as to thereby generate the genetically engineered V3 neuron.

This invention further provides a method of treating a subject having a motor neuron injury or a motor neuron disease comprising: implanting in injured or diseased neural tissue of the subject a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein.

This invention still further provides a method of treating a subject having a motor neuron injury or a motor neuron disease comprising: administering to injured or diseased neural tissue of adult spinal cord a retroviral expression system, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein.

This invention provides a method of treating subject having a motor neuron injury or a motor neuron disease comprising: (a) transfecting neural stem cells with a retroviral vector, which is capable of expressing

homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein; and (b) injecting the transfected neural stem cells of step (a) into the central canal of the spinal cord under conditions which allow the injected transfected neural stem cells to be incorporated into the ependimal layer of the spinal cord.

This invention provides a method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell which comprises: a) contacting a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein with the chemical compound under conditions such that in the absence of the compound the neural stem cell expresses homeodomain transcription factor Nkx6.1 protein and generates a motor neuron; and b) determining what effect, if any, the compound has on generation of the motor neuron.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1B **Homedomain Proteins Define Five Ventral Progenitor Domains**

(Figs. 1A) Localization of homeo-domain proteins in the neural tube of HH stage 20 chick embryos. Class I proteins (Pax7, Dbx2, Irx3, Pax6) have different ventral boundaries (arrowheads). Class II proteins (Nkx6.1 and Nkx2.2) have different dorsal boundaries (arrowheads). The dorsoventral (DV) boundaries of the neural tube are indicated by dotted lines. Composite of expression domains shown in B, p = progenitor domain.

(Figs. 1B) The combinatorial expression of class I and class II proteins defines five ventral progenitor domains. Images show protein expression in the neural tube of HH stage 22 chick embryos.

Figures 2A-2C **Shh Signaling is Required to Establish but not to Maintain the Expression of** Progenitor Homeodomain Proteins.

(Fig. 2A) Repression of class I gene expression by Shh. Expression of Pax7 and *Irx3* in [i] explants grown for 24 h alone or in the presence Shh-N. Repression of Pax7 requires ~1nM Shh-N (Ericson et al., 1996) whereas repression of *Irx3* requires ~3nM Shh-N. Images representative of 12 explants.

(Fig. 2B) Shh induces class II proteins.

Expression of Nkx2.2 and Nkx6.1 in [i] explants exposed to Shh-N for 24 h. Nkx2.2 expression requires ~4nM Shh-N whereas Nkx6.1 expression requires ~ 0.25nM Shh-N. Images representative of 12 explants. (Fig. 2C) Expression of class II proteins requires Shh signaling at stage 10 but not at stage 15. [vf] explants taken from HH stages 10 or 15 embryos grown in the presence of anti-Shh IgG and analyzed for the expression of Nkx2.2, Nkx6.1 and Shh at 24h. Stage 10 [vf] explants grown alone express Nkx2.2 and Nkx6.1. Exposure of stage 10 [vf] explants to anti-Shh IgG blocks the expression of Nkx2.2 and Nkx6.1. Nkx6.1 expression continues in the floorplate of [vf] explants grown in the presence of anti-Shh IgG. Stage 15 [vf] explants grown alone or with anti-Shh IgG express Nkx2.2 and Nkx6.1 in similar domains. The slight narrowing of the domain of Nkx2.2 expression could reflect an influence of Shh on cell proliferation. Anti-Shh IgG blocks Shh signaling effectively in stage 15 [vf] explants (data not shown; see Briscoe et al., 1999). Images representative of 12 explants.

Figures 3A-3O. Repressive Interactions at the pMN/p3 and p1/p2 Boundaries.
Pax6, Nkx2.2, Nkx2.9, Dbx2 and Nkx6.1 were

ectopically expressed using *in ovo* electroporation (*e*) or retroviral transduction (*v*) and the pattern of expression of other progenitor homeodomain proteins was analyzed at HH stages 22-24. (Figs. 3A, 3B) Ectopic expression of Pax6 in the p3 domain results in the cell-autonomous repression of Nkx2.2 A similar level of expression of Pax6 does not repress Dbx2 (data not shown). (Fig. 3C) Number of $Pax6^+$ and $Nkx2.2^+$ cells within the p3 domain of untransfected (left; L) and transfected (right; R) halves of the neural tube (mean ± s.e.m; n=5). (Figs. 3D-3F) Misexpression of Nkx2.2 dorsal to the p3 domain results in the cell-autonomous downregulation of Pax6 (Fig. 3D). Neither Nkx6.1 (Fig. 3E) or Pax7 (Fig. 3F) are repressed by ectopic Nkx2.2 expression. Images representative of 10 embryos. Similar results were obtained after misexpression of *Nkx2.2* by electroporation (not shown). (Figs. 3G-3J) Ectopic expression of Nkx2.9 represses Pax6 expression in a cell-autonomous manner (Fig. 3G). Nkx2.9 does not induce Nkx2.2 expression (Fig. 3H). Nkx2.9 does not repress Pax7 expression (Fig. 3I). Images representative of 10 embryos. (Fig. 3J) Ectopic ventral expression of Dbx2 results in the cell-autonomous repression

of Nkx6.1. Ectopic expression of Dbx2 does not repress Pax6 (Fig. 3K) or Pax7 (Fig. 3L). (Figs. 3M-3O) Misexpression of Nkx6.1 dorsal to the p2 domain represses Dbx2 (Fig. 3M) but not Pax6 (Fig. 3N) or Pax7 (Fig. 3O) expression. Images representative of 10 embryos.

Figures 4A-4L **Each Progenitor Domain Generates a Distinct Neuronal Subtype.** (Figs. 4A-4E) Relationship between class I and class II proteins and neuronal markers. The domain of Nkx6.1 expression encompasses Isl1/2 MNs (Fig. 4A) and Chx10 V2 neurons (Fig. 4C) but is positioned ventral to En1 V1 neurons (Fig. 4D). Chx10 V2 neurons are generated dorsal to HB9 MNs (Fig. 4B). En1 V1 neurons are generated at the ventral extent of the Dbx2 domain (Fig. 4E). Images from HH stage 22-24 embryos. (Figs. 4F-4J) Relationship between class I and class II proteins and neuronal subtype determinants. The domain of Nkx6.1 expression encompasses the domain of generation of Lim3 (Fig. 4F) and MNR2 cells (Fig. 4H). Lim3 cells are positioned ventral to the domain of Dbx2 expression (Fig. 4G). MNR2 cells are positioned ventral to the domain of Irx3 expression (Fig. 4I). Lim1/2 cells derive from Pax6 progenitors (Fig. 4J). (Fig. 4K) The relationship between progenitor domain

identity and neuronal fate. (Fig. 4L) The progenitor homeodomain code within the three ventral-most domains of neurogenesis.

Figures 5A-5C **Nkx6.1 Induces both Motor Neurons and V2 Neurons.**

Patterns of protein expression obtained after misexpression of *Nkx6.1* at rostral (Fig. 5A) and caudal (Fig. 5B) levels of retrovirally-infected embryos. (Fig. 5A) At caudal (lumbar) regions, misexpression of *Nkx6.1* results in ectopic dorsal expression of MNR2 (ii and ix), Lim3 (iii and x), Isl1 (iv and xi), HB9 (v and xii) and Isl2 (vi and xiii). Misexpression of Nkx6.1 induces ectopic Chx10 expression at low incidence and only within the p0 and p1 domain (vii and xiv and data not shown). Electroporation of stage 10 embryos with *Nkx6.1* results in ectopic MNs, at both rostral and caudal levels of the spinal cord (data not shown). (Fig. 5B) In rostral (cervical/thoracic) regions of infected embryos, misexpression of *Nkx6.1* results in the ectopic induction of V2 neurons. Ectopic expression of Chx10 (ix, x, and xi) and Lim3 (vii, viii, x, and xi) is detected ventral to the boundary of Pax7 expression (ix) in the p1 and p0 domains. The misexpression of *Nkx6.1* decreases the number of En1 V1

neurons (xi) and Evx1 V0 neurons (data not shown), but does not induce MNs (xii). Many ectopic Lim3 cells are labeled by a 30 min BrdU pulse, indicating that Nkx6.1 induces Lim3 expression in progenitor cells. Images representative of 10 experiments. (Fig. 5C) The relationship between the domains of ectopic dorsal Nkx6.1 expression , the pattern of expression of *Irx3* at the time of onset of ectopic Nkx6.1 expression, and the fate of neurons that emerge from the domain of ectopic Nkx6.1 expression.

Figures 6A-6F Irx3 Represses Motor Neuron Generation and Induces V2 Neurons. (Fig. 6A) The ventral limit of Irx3 expression corresponds to the dorsal extent of MNR2$^+$ cells in control embryos. Progenitor cells in the ventral-most domain of Irx3 expression give rise to V2 neurons that express Lim3 (Fig. 6B) and Chx10 (Fig. 6C). After ventral misexpression of *Irx3* by electroporation there is no change in the pattern of Lim3 expression (Fig. 6E) but MNR2$^+$ cells are repressed (Fig. 6D) and Chx10$^+$ V2 neurons are generated within the pMN domain (Fig. 6F). Images representative of 10 experiments.

Figures 7A-7B Nkx2.2 Activity Represses Motor Neuron

Generation and Induces V3 Neurons. (Fig. 7A) MNR2$^+$ MN progenitors (i) and HB9$^+$ MNs (ii) are not generated from Nkx2.2 progenitors in control embryos (i and ii). Ectopic expression of Nkx2.2 (iii and iv) in pMN progenitors represses MNR2 (iii) and HB9 (iv) expression. Some more lateral cells coexpress Nkx2.2 and MN markers, probably because cells were infected with *Nkx2.2* virus after they had committed to a MN fate. (Fig. 7B) *Sim1*$^+$ V3 neurons (ii) are generated from Nkx2.2 progenitors (i) in the p3 domain of control embryos. Misexpression of Nkx2.2 (iii) results in the ectopic dorsal expression of *Sim1* (iv). Nkx6.1 (v) has no effect on *Sim1* expression (vi). Nkx2.9 (vii) is sufficient to induce V3 neurons (viii). Images representative of 10 experiments.

Figures 8A-8C **Three Phases of Ventral Neural Patterning.** (Fig. 8A) Graded Shh signaling initiates dorsoventral restrictions in the domains of class I and class II protein expression within the ventral neural tube. Class I proteins are repressed by Shh signals and class II proteins requires Shh signaling. Individual class I and class II proteins have different Shh concentration requirements for repression or activation. (Fig. 8B) Cross-repressive interactions

between class I and class II proteins that abut a common progenitor domain boundary refine and maintain progenitor domains. (Fig. 8C) The profile of expression of class I and class II proteins within an individual progenitor domain controls neuronal fate.

Figures 9A-9U **Selective changes in homeobox gene expression in ventral progenitor cells in *Nkx6.1* mutant embryos.** (Figs. 9A-9C) Expression of *Nkx6.1* in transverse sections of the ventral neural tube of mouse embryos. Expression of *Nkx6.1* is prominent in ventral progenitor cells and persists in some post-mitotic motor neurons at both caudal hindbrain (Fig. 9B) and spinal cord (Fig. 9C) levels. (Fig. 9D, and 9E) Summary diagrams showing domains of homeobox gene expression in wild type mouse embryos (Fig. 9D) and the change in pattern of expression of these genes in *Nkx6.1* mutants (Fig. 9E), based on analyses at e10.0 – e12.5. (Figs. 9F-9I) Comparison of the domains of expression of Nkx6.1, *Dbx2* and *Gsh1* in the caudal neural tube of wild type e10.5 (Figs. 9F, 9G, and 9I) and e12.5 (Fig. 9H) embryos. (Fig. 9J) Absence of Nkx6.1 protein expression in the ventral neural tube of an e10.5 *Nkx6.1* mutant embryo. (Figs. 9K-9M) Change in

pattern of expression of *Dbx2* and *Gsh1* at e10.5 (Figs. 9K and 9M) and of *Dbx2* at e12.5 (9L) in the ventral neural tube of *Nkx6.1* mutant embryos. (Figs. 9N-9Q) Patterns of expression of Sonic hedgehog (Shh) (Fig. 9N), Pax7 (Fig. 9N), Nkx2.2 (Fig. 9O), Pax6 (Fig. 9P), *Dbx1* (Fig. 9P) and *Nkx2.9* (Fig. 9Q) in e10.5 wild type mouse embryos at spinal (Figs. 9N-9P) and caudal hindbrain (Fig. 9Q) levels. Horizontal line in Figs. 9G, 9H, 9K, and 9L indicates approximate position of the dorsoventral boundary of the neural tube, defined by Pax7 expression. Domains of high level Dbx2 and Gsh1 expression are shown by vertical lines in Figs. 9G, 9H, 9K, 9L and 9M. (Figs. 9R-9U). The patterns of Shh (Fig. 9R), Pax7 (Fig. 9R), Nkx2.2 (Fig. 9S), Pax6 (Fig. 9S), *Dbx1* (Fig. 9T) and *Nkx2.9* (Fig. 9U) expression are unchanged in e10.5 *Nkx6.1* mutant embryos. Although the ventral limit of Pax6 expression is not changed in *Nkx6.1* mutant embryos, the level of Pax6 expression by the most ventral progenitor cells is increased (Fig. 9S). Scale bar shown in J= 100μm (Figs. 9A-9C); 50μm (Figs. 9F-9M); 60μm (Figs. 9N-9U).

Figure 10. Disruption of motor neuron differentiation in *Nkx6.1* mutant embryos.
(Fig. 10A-10D) The relationship between

the domain of Nkx6.1 expression by ventral progenitors and the position of generation of motor neurons and V2 interneurons in the ventral spinal cord of e10.5 wild type embryos. (Fig. 10A) Isl1/2 motor neurons (red) are generated within the Nkx6.1 (green) progenitor domain. (Fig. 10B) HB9 motor neurons (red) are generated from the Nkx6.1 (green) progenitor domain. (Fig. 10 C) Lhx3 (Lim3) expression (red) by motor neurons, V2 interneurons and their progenitors is confined to the Nkx6.1 progenitor domain. (Fig. 10D) Chx10 (green) V2 interneurons coexpress Lhx3 (red). (Figs. 10E-10H) Expression of Isl1/2 (Fig. 10E), HB9 (Fig. 10F), Lhx3 (Fig. 10G) and Phox2a/b (Fig. 10H) in the ventral spinal cord (Figs. 10E, 10F, 10G) and caudal hindbrain (Fig. 10H) of e10.5 wild type embryos. At cranial levels, Phox2a/b expression is restricted to visceral motor neurons (Fig. 10H). (Figs. 10I-10L) A perturbation in the differentiation of motor neurons in e10.5 *Nkx6.1* mutant embryos. (Fig. 10I) Few Isl1/2 motor neurons are detected at cervical spinal levels. (Fig. 10J) Few HB9 motor neurons are detected at cervical spinal levels. (Fig. 10K) A marked reduction in Lhx3 expression is detected at upper thoracic levels. (Fig. 10L) There

is a marked reduction in the total number of Isl1/2 motor neurons at upper cervical/ caudal hindbrain levels, but the number of Phox2a/b visceral motor neurons is not decreased. (Figs. 10M-10P) Pattern of expression of Isl1/2 and Lhx3 at cervical (Figs. 10M and 10N) and thoracic (Figs. 10O and 10P) levels of e12.5 wild type embryos. Arrows in Fig. 10M and Fig. 10O indicate the position of Isl1 dorsal D2 interneurons. (Figs. 10Q-10T) Absence of Isl1/2 and Lhx3 expression at cervical levels (Figs. 10Q and 10R) and reduction in Isl1/2 and Lhx3 expression at thoracic levels (Figs. 10S and 10T) in e12.5 *Nkx6.1* mutant embryos. Scale bar shown in I = 60μm (Figs. 10A-10D); 80μm (Figs. 10E-10L); 120μm (Figs. 10M-10T).

Figures 11A-11J **Motor neuron subtype differentiation in *Nkx6.1* mutant mice.** (Figs. 11A and 11B) Depletion of both median motor column (MMC) and lateral motor column (LMC) neurons in *Nkx6.1* mutant mice. Sections of e12.5 wild type (Fig. 11A) and *Nkx6.1* mutant (Fig. 11B) mice spinal cord at forelimb levels show coexpression of Lhx3 (green) and Isl1/2 (red) in MMC (yellow) neurons and expression of Isl1/2 alone in LMC neurons. Both columnar subclasses of motor neurons are depleted in *Nkx6.1* mutant mice. (Figs.

11C and 11D) *RALDH2* expression by LMC neurons in e12.5 forelimb level spinal cord of wild type (Fig. 11C) and *Nkx6.1* mutant (Fig. 11D) mice. (Figs.11E-11J) Motor neuron generation at caudal hindbrain (rhombomere [r] 7/8) level. (Figs. 11E and 11F) Pattern of Nkx6.1 expression in progenitor cells and visceral motor neurons in the caudal hindbrain of e10.5-e11 wild type mice (Fig. 11E) and absence of protein expression in *Nkx6.1* mutant mice (Fig. 11F). (Figs. 11G and 11H) HB9 expression in hypoglossal motor neurons in e10.5-e11 wild type mice (Fig. 11G) is lacking in *Nkx6.1* mutant mice (Fig. 11H). (Figs. 11I and 11J) In e10.5-e11 wild type mice (Fig. 11I) visceral vagal motor neurons (v) coexpress Isl1 (green) and Phox2a/b (red) whereas hypoglossal motor neurons (h) lack Phox2a/b expression. In e11 *Nkx6.1* mutant mice (Fig. 11J) visceral vagal motor neurons (v) persist in normal numbers but hypoglossal motor neuronal are absent. Scale bar shown in C = 50μm (Figs. 11A-11D); 70μm (Figs. 11E-11J).

Figures 12A-12L **A switch in ventral interneuron fates in *Nkx6.1* mutant mice.**

(Figs. 12A and 12B) Chx10 expression in V2 neurons at rostral cervical levels of an e10.5 wild type embryo (Fig. 12A) and the

absence of expression of Chx10 in *Nkx6.1* mutant embryos (Fig. B12). (Figs. 12C and 12D) Expression of En1 by V1 neurons at rostral cervical levels of an e10.5 wild type embryo (Fig. 12C) and the ventral expansion of the domain of V1 neuron generation in *Nkx6.1* mutant embryos (Fig. 12D). (Figs. 12E and 12F) Pax2 expression in a set of interneurons that includes V1 neurons (21) at caudal hindbrain levels of an e10.5 wild type embryo (Fig. 12E) and the ventral expansion of the domain of Pax2 expression in *Nkx6.1* mutant embryos (Fig. 12F). (Figs. 12G and 12H) Expression of *Sim1* by V3 neurons in the cervical spinal cord of an e10.5 wild type (Fig. 12G) and *Nkx6.1* mutant (Fig. 12H) embryos. (Figs. 12I and 12J) Expression of *Evx1* by V0 neurons at caudal hindbrain levels of e10.5 wild type (Fig. 12I) and *Nkx6.1* mutant (Fig. 12J) embryos. (Fig. 12K and 12L) En1 (red) and Lhx3 (green) expression by separate cell populations in the ventral spinal cord of e11 wild type embryos (Fig. 12K). In *Nkx6.1* mutant embryos (Fig. 12L) coexpression of En1 and Lhx3 is detected in many cells within the normal domain of V2 neuron generation. Scale bar shown in B = 60μm (Figs. 12A-12D); 75μm (Figs. 12E, 12F); 70μm (Figs. 12G, 12J, 12H, 12J), 35μm (Figs. 12K and

12L).

Figure 13A-13B Summary of changes in progenitor domain identity and neuronal fate in the spinal cord of *Nkx6.1* mutant embryos. (Fig. 13A). In wild type mouse embryos, cells in the *Nkx6.1* progenitor domain give rise to three classes of ventral neurons: V2 neurons, motor neurons (MN) and V3 neurons. V3 neurons derive from cells in the ventral most region of *Nkx6.1* expression that also express *Nkx2.2* and *Nkx2.9*. V1 neurons derive from progenitor cells that express *Dbx2* but not *Nkx6.1*. (Fig. 13B). In *Nkx6.1* mutant embryos the domain of Dbx2 expression by progenitor cells expands ventrally, and by e12 occupies the entire dorsoventral extent of the ventral neural tube, excluding the floor plate. Checked area indicates the gradual onset of ventral *Dbx2* expression. This ventral shift in *Dbx2* expression is associated with a marked decrease in the generation of V2 neurons and motor neurons and a ventral expansion in the domain of generation of V1 neurons. The generation of V3 neurons (and cranial visceral motor neurons at hindbrain levels) is unaffected by the loss of *Nkx6.1* or by the ectopic expression of *Dbx2*.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a genetically engineered cell

comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein. In an embodiment of the above-described genetically engineered stem cell, the neural stem cell is a mammalian neural stem cell. In a preferred embodiment, the mammalian stem cell is a human neural stem cell.

This invention provides a method of generating a genetically engineered motor neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein so as to thereby generate the genetically engineered motor neuron. In an embodiemnt of the above-described method of generating a genetically engineered motor neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein, the neural stem cell

is a mammalian cell neural stem cell. In a preferred embodiment, the mammalian neural stem cell is a human neural stem cell.

This invention provides a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein. In an embodiemnt of the above-described genetically engineered stem cell, the neural stem cell is a mammalian neural stem cell. In a preferred embodiment of the genetically engineered cell, wherein the mammalian neural stem cell is a human neural stem cell.

This invention provides a method of generating a genetically engineered V2 neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein, under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein so as to thereby generate the genetically engineered V2 neuron. In an embodiment of the above-described method of generating a genetically engineered V2 neuron which is capable of expressing

homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein, the neural stem cell is a mammalian neural stem cell. In a preferred embodiment, the mammalian neural stem cell is a human neural stem cell.

This invention provides a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein. In an embodiment of the above-described genetically engineered cell the neural stem cell is a mammalian neural stem cell. In a preferred embodiment, the mammalian neural stem cell is a human neural stem cell.

This invention provides a method of generating a genetically engineered V3 neuron which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein which comprises treating a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein, under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein so as to thereby generate the genetically engineered V3 neuron. In an embodiment of the above-

described method of generating a genetically engineered V3 neuron which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein, the neural stem cell is a mammalian neural stem cell. In a preferred embodiment, the mammalian neural stem cell is a human neural stem cell.

In the practice of the methods described herein one of skill may use any suitable retroviral vector t express the desired protein(s).

This invention provides a method of treating a subject having a motor neuron injury or a motor neuron disease comprising: implanting in injured or diseased neural tissue of the subject a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein. In an embodiment of the above-described method of treating subject having a motor neuron injury or a motor neuron disease the neural stem cells are transfected with the retroviral expression system ex vivo and implanted into the subject. In another embodiment of the above-described method the neural stem cells are transfected with the retroviral expression system in vitro and implanted into the subject. In a further embodiment of the above-described method the motor neuron disease is amyotrophic lateral sclerosis (AML), spinal

muscular atrophy(SMA) or any motor neuron degenerative disease. In a preferred embodiment of the above-described method the neural stem cells are from the developing mammalian nervous system. In another preferred embodiment of the above-described method the neural stem cells are from the adult mammalian nervous system. The nervous system may be from any mammal including human. The genetically engineered implanted cells will express homeodomain transcription factor Nkx6.1 protein and thereby generate motor neurons. The genetically engineered implanted cells may also affect endogenous neural stem cells into generating motor neurons.

This invention provides a method of treating a subject having a motor neuron injury or a motor neuron disease comprising: administering to injured or diseased neural tissue of adult spinal cord a retroviral expression system, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein. In an embodiment of the above-described method of treating subject having a motor neuron injury or a motor neuron disease the motor neuron injury may be a spinal cord injury. In another embodiment of the above-described method the motor neuron disease is amyotrophic lateral sclerosis, spinal muscular atrophy (SMA) or any other motor neuron degenerative disease. The retroviral expression system will express homeodomain transcription factor Nkx6.1 protein and thereby generate motor neurons in endogenous neural stem cells of the adult spinal cord or in the

injured or diseased neural tissue of adult spinal cord.

This invention provides a method of treating a subject having a motor neuron injury or a motor neuron disease comprising: a) transfecting neural stem cells with a retroviral vector, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein; and b) injecting the transfected neural stem cells of step (a) into the central canal of the spinal cord under conditions which allow the injected transfected neural stem cells to be incorporatedinto the ependimal layer of the spinal cord. In an embodiment of the above-described method the neural stem cells are from the developing mammalian nervous system. In a preferred embodiment, the neural stem cells are from the adult mammalian nervous system. The subject may be any mammal including a human. In the above-described method the transfected neural stem cells will generate motor neurons in the ependimal layer of the spinal cord which are in/near the central canal.

Since neural stem cells exist not only in the developing mammalian nervous system but also in the adult nervous system of all mammalian organisms, including humans (see Gage, F.H., Science 287:1433- (2000)), the above-described method is useful in any stem cell based therapy to control the neural cell types that generated by a stem cell to ensure replacement of the appropriate cells or repair of injured cells. For example, any of the above-described genetically engineered cells may be trans-

planted into a human suffering from a neurodegenerative disease (including but not limited to ALS or SMA) or injuries in the nervous system, e.g. spinal cord, to replace missing or injured cells in the subject or to repair endogenous stem cells in the subject, e.g. neural stem cells genetically engineered to produce motor neurons by expression of the appropriate homeodomain protein code in vivo or ex vivo. (see also Doetsch, F. et al. (1999) Cell 97(6):703-716 and Johansson C. B. et al. (1999) Cell 96(1):25-34) Any of the above-described genetically engineered cell lines, especially motor neurons, are also useful for in vivo or in vitro studies in pharmaceutical assays to determine which compounds which induce, increase, decrease, or inhibit generation of a motor neuron from a neural stem cell.

One of skill is familiar with techniques which introduce stem cells into the spinal cord, as well as conditions under which the introduced stem cells will performed the desired protein expression, such as those used in treatment of Parkinson's disease. Techniques and conditions such as these may be implemented in the practice of the methods described herein.

The genes studied herein, including *Nkx6.1* which encodes homeodomain transcription factor Nkx6.1 protein, are highly conserved in mammalian cells. Therefore, the experiments set forth herein are the basis of genetic engineering of human neural stem cells (progenitor cells) to enable generation of motor neurons, or V2 and V3 neurons, which are used in motor control, in the

treatment of motor neuron degenerative diseases or neural disease in which the genes encoding the proteins required for their generation are either missing or mutated.

This invention provides a method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell which comprises: a) contacting a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein with the chemical compound under conditions such that in the absence of the compound the neural stem cell expresses homeodomain transcription factor Nkx6.1 protein and generates a motor neuron; and b) determining what effect, if any, the compound has on generation of the motor neuron. In an embodiment of the above-described method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell the chemical compound promotes generation of the motor neuron. In another embodiment of the above-described method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell the chemical compound inhibits generation of the motor neuron.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific

methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

EXPERIMENTAL DETAILS

First Series of Experiments

Experimental Procedures

Retroviral Transduction and *In Ovo* Electroporation

Mouse *Nkx2.2*, *Nkx2.9*, *Nkx6.1*, chick *Dbx2* and *GFP* cDNAs were cloned into RCASBP(A) and (B) vectors (Hughes et al., 1987; Morgan and Fekete, 1996). Viral supernatants (Morgan and Fekete, 1996) were applied to Hamburger-Hamilton (1951) (HH) stage 5-6 chick embryos in ovo. Retroviral transduction resulted in expression of the target protein 12-14 h post-infection (data not shown). For electroporation cDNAs were cloned into RCASBP or pNES (gift of U.Lendhal) vectors. HH stage 10-12 chick embryos were electroporated unilaterally with cDNAs for mouse *Irx3*, *Pax6*, *RCASBP(Dbx2)* and *RCASBP(GFP)* using a T820 electro-squareporator (BTX Inc) and ectopic protein expression was detected after 2-4 h. Embryos were analyzed at HH stages 20-24.

Immunocytochemistry and In Situ Hybridization Histochemistry

Guinea-pig antisera were generated against peptides encoding the N-terminal 14 residues of mouse Irx3 and the N-terminal 12 residues of mouse Nkx2.9. Other antibody

reagents and protocols have been described (Yamada et al., 1993; Ericson et al, 1997a; Tanabe et al., 1998; Pierani et al., 1999; Briscoe et al., 1999). In situ hybridization was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993), using probes for *Irx3*, *Nkx2.2*, *Sim1*, *Nkx6.1* and *Nkx2.9* (Briscoe et al., 1999).

BrdU Incorporation

To define mitotic cells, 100μM BrdU was applied to HH stage 22 embryos, followed by incubation at 37°C for 30 min, at which time embryos were fixed and analyzed.

Neural Explant Culture

Neural explants were isolated from intermediate [i] regions of stage 10 chick neural plate or ventral + floor plate [vf] regions from stage 10 or stage 15 embryos, as described (Yamada et al., 1993; Ericson et al., 1996). Explants were cultured for 24 h with or without Shh-N (Ericson et al., 1996), or in the presence of anti-Shh IgG (20μg/ml; Ericson et al., 1996). Explants were processed as described (Ericson et al., 1997a).

Results

A Homeodomain Protein Code for Ventral Progenitor Cells

Shh signaling controls the generation of five distinct classes of neurons, each at a different dorsoventral position in the ventral neural tube (Briscoe et al., 1999; Ericson et al., 1997a; Pierani et al., 1999). The spatial information provided by the five homeodomain proteins examined previously - Pax7, Dbx1, Dbx2, Pax6 and Nkx2.2 - is not sufficient to establish distinct progenitor domains for each post-mitotic neuronal subtype (Ericson et al., 1996; Ericson et al., 1997a; Briscoe et al., 1999; Pierani et al., 1999), prompting a search for other relevant homeodomain proteins. It was found that two additional proteins, Nkx6.1 (Qiu et al., 1998) and Irx3 (Funayama et al., 1999), are expressed by distinct sets of ventral progenitor cells.

Compared were the patterns of expression of Nkx6.1 and Irx3 with the homeodomain proteins characterized previously. The combinatorial expression of this set of seven homeodomain proteins is sufficient to define five ventral progenitor cell (p) domains, which are termed the p0, p1, p2, pMN and p3 domains, in dorsal-to-ventral progression (Figure 1A). The ventral limit of Pax7 expression defines the dorsal/p0 boundary (Figure 1Bi; Ericson et al., 1996); the ventral limit of Dbx1 expression defines the p0/p1 boundary (Figure 1Bii; Pierani et al., 1999); the ventral limit of Dbx2 expression defines the p1/p2 boundary (Figure 1Biii; Pierani et al., 1999); the ventral limit of Irx3

expression defines the p2/pMN boundary (Figure 1Biv, vi); and the ventral limit of Pax6 expression defines the pMN/p3 boundary (Figure 1Bv, vii; Ericson et al., 1997a). The dorsal limit of Nkx6.1 expression complements the ventral limit of Dbx2 expression at the p1/p2 boundary (Figure 1Biii); and the dorsal limit of Nkx2.2 expression complements the ventral limit of Pax6 expression at the pMN/p3 boundary (Figure 1Bvii; Ericson et al., 1997a).

These seven homeodomain proteins can therefore be divided into two major subclasses. Five proteins - Pax7, Dbx1, Dbx2, Irx3 and Pax6 - exhibit ventral limits of expression that delineate progenitor domain boundaries, and these are termed class I proteins (Figure 1A). Two proteins - Nkx6.1 and Nkx2.2 - exhibit dorsal limits of expression that define progenitor domain boundaries, and these are termed class II proteins (Figure 1A).

Progenitor Homeodomain Protein Expression is Initiated by an Early Period of Graded Sonic Hedgehog Signaling

The expression of certain class I (Pax7, Dbx1, Dbx2, Pax6) and class II (Nkx2.2) proteins is controlled by Shh signaling in vitro (Ericson et al., 1996; Ericson et al., 1997a; Briscoe et al., 1999; Pierani et al., 1999). The expression of class I proteins is repressed by Shh signaling, and the more ventral the boundary of class I protein expression in vivo, the higher is the concentration of Shh required for repression of protein expression in vitro (Ericson et al., 1997a). Conversely, Shh signaling is required to induce expression of the class II protein Nkx2.2 in vitro (Briscoe et al., 1999;

Ericson et al., 1997a).

Examined was whether this relationship extends to Irx3 and Nkx6.1 by assaying the expression of these two proteins in intermediate neural plate [i] explants exposed to different Shh-N concentrations. Repression of Irx3 required ~3nM Shh-N (Figure 2A), a concentration greater than that required for repression of Pax7, Dbx1 and Dbx2 expression (Figure 2A; Ericson et al., 1996; Pierani et al., 1999), but less than that required for complete repression of Pax6 (Ericson et al., 1997a). Conversely, induction of Nkx6.1 required ~0.25nM Shh-N; a concentration lower than that required for induction of Nkx2.2 (3-4nM; Ericson et al., 1997a; Figure 2B). Thus, the link between the domains of expression of class I and class II proteins in vivo and the Shh concentration that regulates their expression in vitro extends to Irx3 and Nkx6.1 (Figure 2A, B). These findings support the idea that the differential patterns of expression of all class I and class II proteins depend initially on graded Shh signaling.

Next asked was whether Shh signaling is required continuously to maintain the early pattern of progenitor homeodomain protein expression. To address this examined was whether the expression of class II proteins, once initiated, can be maintained under conditions in which ongoing Shh signaling is eliminated. Explants of ventral neural tube, including the floor plate, ([vf] explants) were isolated from stage 10 or stage 15 embryos and grown in vitro, alone or in the presence of a function blocking

anti-Shh antibody (Ericson et al., 1996). Both stage 10 and stage 15 [vf] explants grown alone generated a narrow domain of Nkx2.2$^+$ cells and a broad domain of Nkx6.1$^+$ cells (Figure 2Ci, ii, v, vi). Addition of anti-Shh IgG to stage 10 [vf] explants blocked the expression of both Nkx2.2 and Nkx6.1 in neural progenitors (Figure 2Ciii, iv). In contrast in stage 15 [vf] explants, the domains of Nkx2.2 and Nkx6.1 expression persisted in the presence of anti-Shh IgG (Figure 2Cvii, viii). These results provide evidence that the pattern of class II protein expression becomes independent of Shh signaling over a period of ~12-15h, between stages 10 and 15.

Cross-Repressive Interactions Between Class I and Class II Proteins Refine Progenitor Domain Boundaries

The boundaries of progenitor domains are sharply delineated in vivo (Figure 1), raising questions about the steps that operate downstream of Shh signaling to establish the non-graded domains of expression of class I and class II proteins. Examined was whether the domain of expression of class I proteins might be constrained by the action of the class II protein that abuts the same domain boundary, and vice versa. To test this, individual homeodomain proteins in the chick neural tube were misexpressed in mosaic fashion, and the resulting pattern of class I and class II protein expression was assayed. Ectopic protein expression was achieved using either retroviral transduction or electroporation.

Interactions at the p3/pMN boundary

First analyzed was the interaction between the class I protein Pax6 and the class II protein Nkx2.2 -- proteins

that exhibit complementary domains of expression at the pMN/p3 boundary. To assess the influence of Pax6 on Nkx2.2, Pax6 was misexpressed ventral to its normal limit and the resulting pattern of expression of Nkx2.2 was examined (Figure 3A-C). After electroporation of *Pax6*, small clusters of ectopic Pax6$^+$ cells were detected within the p3 domain (Figures 3A, 3B). These cells lacked Nkx2.2 expression (Figures 3A, 3B), whereas expression of Nkx2.2 was maintained by neighboring p3 domain cells that lacked ectopic Pax6 expression (Figures 3A, 3B), arguing for a cell-autonomous action of Pax6. The expression of other class I and class II proteins was not affected by the deregulated expression of Pax6 (data not shown). Thus, Pax6 acts selectively to repress Nkx2.2 expression in p3 domain cells. These results complement studies showing a requirement for Pax6 activity in defining the dorsal limit of the p3 domain in vivo (Ericson et al., 1997a).

To examine whether Nkx2.2 normally limits the ventral boundary of Pax6 expression, Nkx2.2 was misexpressed in regions dorsal to the p3 domain. The vast majority (>95%) of progenitor cells that ectopically expressed Nkx2.2 lacked Pax6 expression (Figure 3D). Since these experiments used a replication competent retroviral expression system, the coexpression of both homeodomain proteins in a small minority of cells is likely to reflect the secondary infection of cells at later stages, with the consequence that Nkx2.2 may be expressed for too brief a period to repress Pax6 completely. Neighboring cells that lacked ectopic Nkx2.2 retained Pax6 expression

(Figure 3D), indicating a cell-autonomous action of Nkx2.2. The expression of Nkx6.1 and Pax7 was unaffected by the ectopic expression of Nkx2.2 (Figures 3E, 3F). Thus, the repressive action of Nkx2.2 on Pax6 expression is selective and cell-autonomous. These results provide evidence for mutually repressive interactions between Pax6 and Nkx2.2 at the pMN/p3 boundary.

Nkx2.9, a gene closely related to *Nkx2.2* (Pabst et al., 1998), is expressed in a pattern that overlaps transiently with *Nkx2.2* in the p3 domain (Briscoe et al., 1999). To test whether these two genes have similar activities, *Nkx2.9* was expressed ectopically and the pattern of Pax6 expression was examined. Most (>95%) cells that expressed Nkx2.9 ectopically lacked Pax6 expression (Figure 3G). Moreover, the repression of Pax6 occurred in the absence of Nkx2.2 induction (Figure 3H), showing that Nkx2.9 acts independently of Nkx2.2. Thus, Nkx2.2 and Nkx2.9 have similar abilities to repress Pax6 expression and are likely to act in parallel in defining the ventral boundary of the pMN domain in vivo (Briscoe et al., 1999).

Interactions at the p1/p2 boundary

Next examined was whether cross-regulatory interactions occur between the class I protein Dbx2 and the class II protein Nkx6.1 — proteins with complementary domains of expression at the p1/p2 boundary. First Dbx2 was misexpressed in regions ventral to the p1 domain and the pattern of homeodomain protein expression was monitored. Most (>95%) ventral cells that ectopically expressed Dbx2

lacked expression of Nkx6.1 (Figure 3J), whereas neighboring cells that lacked Dbx2 maintained Nkx6.1 expression (Figure 3J). Misexpression of Dbx2 did not alter the expression of Pax6 or Pax7 (Figures 3K, 3L). Thus, the repressive action of Dbx2 is selective and cell-autonomous. Also examined was the consequences of misexpression of Nkx6.1 on the expression of Dbx2. Most (>95%) progenitor cells that ectopically expressed Nkx6.1 lacked Dbx2 expression (Figure 3M), whereas neighboring cells that lacked ectopic Nkx6.1 maintained Dbx2 expression (Figure 3M). Ectopic expression of Nkx6.1 did not repress Pax6 or Pax7 (Figures 3N, 3O). Thus, Nkx6.1 acts selectively and in a cell-autonomous manner to repress Dbx2 expression.

These results reveal that the two pairs of class I and class II proteins that share a common progenitor domain boundary exhibit mutual repressive interactions. Such interactions are likely to contribute to the establishment and sharp delineation of progenitor domain boundaries evident in vivo.

The Relationship Between Progenitor Domain and Neuronal Fate

Next examined was the relationship between the five progenitor domains defined by class I and class II protein expression and the pattern of neurogenesis in the ventral neural tube. It was found previously that Evx1/2$^+$ V0 neurons derive from cells within the p0 domain (see Pierani et al., 1999; Ericson et al., 1997a), that En1$^+$ V1 neurons derive from cells within the p1 domain (Ericson et al., 1997a; Pierani et al., 1999) (Figures 4D and 4E)

and that *Sim1*$^+$ V3 neurons derive from cells within the p3 domain (Briscoe et al., 1999). It is shown here that Chx10$^+$ V2 neurons derive exclusively from cells within the p2 domain (Figures 4B and 4C) (Ericson et al., 1997a) and that HB9$^+$ motor neurons (MNs) derive only from cells within the pMN domain (Figures 4A and 4B) (Tanabe et al., 1998). Thus, a precise register exists throughout the neural tube between the dorsoventral extent of individual ventral progenitor domains and the position at which specific neuronal subtypes are generated.

Progenitor cells express a separate set of homeodomain proteins at late stages in the pathway of ventral neurogenesis. The final division of V2 neuron and MN progenitors is accompanied by the onset of expression of Lim3 (Ericson et al., 1997a; Sharma et al., 1998; Tanabe et al., 1998). Late stage MN progenitors express MNR2 (Tanabe et al., 1998). Lim3 and MNR2 appear to function respectively as determinants of V2 neuron and MN identity (Sharma et al., 1998; Tanabe et al., 1998). Therefore, examined was whether the expression of Lim3 and MNR2 also conforms to the domains defined by class I and class II protein expression. Lim3 expression was excluded from the p0 and p1 domains but was detected within both the p2 and pMN domains (Figures 4F and 4G and data not shown), whereas MNR2 expression was confined to the pMN domain (Figures 4H-4J). Thus, the expression of these two ventral neuronal subtype determinants also respects progenitor domain subdivisions defined by class I and class II protein expression. The concordance in expression of progenitor homeodomain proteins, late stage

progenitor determinants and neuronal fate supports the idea that the subdivision of the neural epithelium into five progenitor domains is a fundamental step in the allocation of cell fate in the ventral neural tube.

Nkx6.1 Activity Directs Motor Neuron and V2 Neuron Generation

If the combinatorial expression of class I and class II proteins within progenitor cells directs the fate of ventral neurons, then changing the expression profile of these proteins would be expected to alter patterns of neurogenesis. The analysis of this issue was focused on the three ventral-most progenitor domains, from which V2 neurons, MNs and V3 neurons are generated (Figure 4K). The combinatorial expression of Nkx6.1, Irx3 and Nkx2.2 distinguishes these three domains of neurogenesis (Figure 4L), and poses three questions about their role in the assignment of neuronal subtype identity. First, is whether the expression of Nkx6.1 in the absence of expression of Irx3 or Nkx2.2/Nkx2.9 sufficient to result in the generation of MNs. Second, is whether the coincidence in expression of Nkx6.1 and Irx3 result in the generation of V2 neurons, at the expense of MNs. Third, is whether the expression of Nkx2.2/Nkx2.9 and Nkx6.1 result in the generation of V3 neurons rather than MNs.

To test whether Nkx6.1 activity is able to generate MNs, a way of misexpressing Nkx6.1 in neural progenitor cells in the absence of high level *Irx3* expression was searched. All progenitor cells dorsal to the p2/pMN boundary express *Irx3* (data not shown). The onset of *Irx3*

expression occurs only after neural tube closure, later than that of Nkx6.1 and is excluded from the ventral-most region of the neural tube (Supplemental Figure S1; available at http://www.cell.com/cgi/content/full/101/4/[redacted]/DC1)). It was reasoned therefore that misexpression of *Nkx6.1* by dorsal neural cells, prior to the onset of *Irx3* expression, might establish an initial homeodomain protein code (Nkx6.1$^+$, Irx3$^-$) that mimics that found normally in the pMN domain, and thus lead to ectopic MN generation.

Two approaches were taken to achieve early ectopic expression of Nkx6.1. First, *Nkx6.1* was misexpressed in stage 5-6 embryos by retroviral transduction (Figure S1A). With this method the onset of ectopic protein expression occurs about 12-16 h later, at approximately stages 12-14 (Figure S1B). At this stage, only at the most caudal levels of infected embryos was ectopic neural expression of Nkx6.1 detected before the onset of expression of *Irx3* (Figure S1C). At more rostral levels, the onset of ectopic protein expression occurs at a stage when neural cells already express *Irx3* (Figure S1D). *Nkx6.1* was also misexpressed by electroporation in stage 10 embryos (Figure S1E). In this case, expression of transgenes was detected within ~2-4 h (Figure S1F; Muramatsu et al., 1997). Under these conditions, *Nkx6.1* was expressed ectopically prior to the onset of *Irx3* expression over a broader rostrocaudal region of the neural tube (Figures S1F-S1H). Based on these observations, embryos that had been retrovirally infected or electroporated *in ovo* with *Nkx6.1* constructs were

permitted to develop until stages 22-24, and the resulting pattern of neurogenesis was examined.

First examined were levels of the neural tube where ectopic dorsal neural expression of Nkx6.1 occurred prior to that of endogenous Irx3. At these levels, the MN subtype determinants MNR2 and Lim3 were detected in ectopic dorsal positions, in both progenitor cells and post-mitotic neurons (Figures 5A ix and 5Ax and data not shown). In addition, ectopic dorsal expression of the post-mitotic MN markers Isl1, Isl2 and HB9 was detected (Figures 5Axi-5Axiii and data not shown). The ectopic expression of Isl1, Isl2 and HB9 was, however, limited to post-mitotic MNs located in the lateral margin of the neural tube (Figures 5Axi-5Axiii). This finding is consistent with previous studies documenting that MNR2 can induce these MN markers only after cells have acquired post-mitotic status (Tanabe et al., 1998). Strikingly, the expression of MN markers was detected both dorsal to the p2 domain boundary in the ventral neural tube, and throughout the dorsal extent of the neural tube (Figure 5A and data not shown). Under these conditions, additional ectopic Chx10$^+$ V2 neurons were occasionally detected within the p0 and p1 domains, but were not detected in the dorsal spinal cord (Figure 5A xiv and see below). These results show that misexpression of Nkx6.1 in neural cells at stages before the onset of *Irx3* expression can induce ectopic MN generation (Figure 5C).

Next examined was the fate of cells at levels of the

neural axis where ectopic expression of Nkx6.1 occurred together with *Irx3*. Misexpression of Nkx6.1 at this level resulted in the ectopic generation of many Chx10$^+$ V2 neurons within the p0 and p1 domains (Figures 5Bix-5Bxi). Many ectopic Lim3$^+$ cells were also detected within these domains, some of which were mitotic progenitors (Figure 5B viii). In addition, the ectopic expression of Nkx6.1 within the p0 and p1 domains resulted in a marked decrease in the number of En1$^+$ V1 (Figure 5Bxi) and Evx1/2$^+$ V0 neurons (data not shown). Ectopic MN markers were not detected, suggesting that the coincident expression of *Irx3* attenuates the ability of Nkx6.1 to induce MNs (Figure 5B xii). Together, these results support the idea that Nkx6.1, in the context of *Irx3* activity, promotes the generation of V2 neurons (Figure 5C).

Misexpression of Irx3 Directs V2 Neuron Generation at the Expense of Motor Neurons

To test more directly whether the expression of Irx3 in progenitor cells that express Nkx6.1 results in a switch from MN to V2 neuron fate, Irx3 was misexpressed in regions ventral to the p2 domain and the resulting pattern of neurogenesis was examined. Cells that ectopically expressed Irx3 failed to express the MN markers MNR2, Isl1/Isl2 or HB9 (Figures 6A and 6D and data not shown). Neighboring pMN cells that lacked ectopic Irx3 expression maintained expression of these MN markers (Figure 6D), indicating the cell-autonomy of Irx3 action. In addition, V2 neurons, defined by Chx10 expression, were generated at markedly more ventral positions, within the normal domain of MN generation

(Figures 6C and 6F). The pattern of Lim3 expression was not altered by ventral misexpression of Irx3 (Figure 6B and 6E), consistent with the normal overlap of Lim3 and Irx3 expression within the p2 domain.

These findings, taken together with the results of late Nkx6.1 misexpression described above, indicate that coexpression of Irx3 and Nkx6.1 by ventral progenitor cells specifies V2 neuron identity. The domain of the ventral neural tube in which Nkx6.1 is able to generate MNs thus appears to be limited by the expression of Irx3 in cells dorsal to the p2/pMN domain boundary.

Nkx2.2 Constrains the Ability of Nkx6.1 to Induce Motor Neurons

Next examined was whether the expression of Nkx2.2 within the pMN domain is sufficient to repress MN generation. To test this Nkx2.2 was misexpressed in regions dorsal to the p3 domain and the resulting pattern of neurogenesis was examined. Detected was a marked repression in the expression of MNR2, Lim3, Isl1, Isl2 and HB9 in cells that expressed Nkx2.2 (Figure 7A and data not shown). A few ectopic Nkx2.2-labeled cells that co-expressed HB9 were detected in a lateral position, characteristic of post-mitotic neurons (Figure 7A). The coexpression of Nkx2.2 and MN markers in these cells is likely to reflect the late onset of expression of Nkx2.2, after cells have committed to a MN fate. These results show that Nkx2.2 activity is sufficient to repress MN differentiation, extending findings that Nkx2.2 activity within the p3 domain is required to suppress MN fate (Briscoe et al., 1999).

Nkx2.2 Expression Directs V3 Interneuron Generation

The role of Nkx2.2 in repressing MN generation raised the additional issue of whether Nkx2.2 activity is sufficient to generate V3 neurons. To test this the pattern of expression of the V3 neuron marker *Sim1* in *Nkx2.2*-infected embryos was analyzed. Misexpression of Nkx2.2 directed the ectopic expression of *Sim1* both within the domain of Nkx6.1 expression and throughout the dorsal neural tube (Figures 7Bi-7Biv). Nkx2.2 did not induce ectopic Nkx6.1 expression (data not shown), and Nkx6.1 was not sufficient to induce V3 neurons (Figures 7Bv and 7Bvi). Thus, Nkx2.2 is able to induce V3 neurons independently of Nkx6.1 activity. Nkx2.9 mimicked the ability of Nkx2.2 to induce V3 neurons (Figures 7Bvii and 7Bviii), supporting the idea that these two proteins have equivalent patterning activities. These findings, taken together with studies of *Nkx2.2* mutant mice (Briscoe et al., 1999), establish the critical role of Nkx2.2 in suppressing MN and promoting V3 neuron fates.

Discussion

The results described in this series of experiments fit most easily into a three step model that links graded Shh signaling, the expression of class I and class II proteins by neural progenitor cells and the pattern of neuronal subtype generation in the ventral neural tube (Figure 8). In a first step, the expression of progenitor cell homeodomain proteins is differentially repressed or activated by graded Shh signaling (Figure 8A). In a second step, cross-repressive interactions

between class I and class II proteins establish, refine and stabilize progenitor domains (Figure 8B). In a third step, the profile of homeodomain proteins expressed within each progenitor domain directs the generation of specific sets of post-mitotic neurons (Figure 8C). Each step of this model is discussed in the context of the interpretation of graded extracellular signals during the patterning of embryonic tissues.

Formation and Maintenance of Neural Progenitor Domains

The findings herein address first the issue of how discrete progenitor domains are established in the ventral neural tube, in response to Shh signaling. A ventral to dorsal gradient of Shh signaling activity appears to have an initial role in defining the dorsoventral domains over which individual class I and class II proteins are expressed. Yet, the existence of an extracellular gradient of Shh activity does not offer an easy explanation for the sharp boundaries that exist between progenitor domains. These findings suggest that cross-repressive interactions that occur between class I and class II proteins may serve two early roles: first to establish the initial dorsoventral domains of class I and class II protein expression, and second to refine the initially imprecise pattern of homeodomain protein expression initiated by graded Shh signals. Support for this idea comes from the analysis of ventral patterning in mouse mutants lacking homeodomain protein function. The loss of Pax6 function leads to an expansion in the dorsoventral extent of the p3 domain, despite a constant level of Shh activity (Ericson et al., 1997a).

Conversely, the loss of Nkx6.1 function results in a ventral expansion in the extent of the p1 domain, without any change in Shh signaling (Sander et al., submitted). It is noteworthy that the boundaries of each of the five progenitor domains are sharply defined, yet class II proteins have been identified only at the pMN/p3 and p1/p2 boundaries. Thus, additional class II proteins may exist, with patterns of expression that complement the three orphan class I proteins.

A second issue is how individual progenitor domains are maintained in relatively constant proportions over time. As neuronal fates are established, ventral progenitor cells undergo multiple rounds of proliferation (Langman et al., 1966) and the dorsoventral extent of the ventral neural tube increases markedly in size. Thus, the level of Shh activity at a given position in the ventral neural tube is likely to change significantly over time. The findings herein show that by stage 15, ventral progenitor domains can be maintained despite the loss of Shh signaling. The cross-repressive interaction between class I and class II proteins may help to maintain progenitor domains over time, in the face of a changing level of Shh activity. The findings suggest that these cross-repressive interactions relieve progenitor cells of a requirement for ongoing Shh signaling but do not exclude that Shh has a later role in regulating the proliferation of cells within individual progenitor domains (Rowitch et al., 1999).

How do neural progenitor cells initially perceive the

extracellular gradient of Shh activity? Several components of the vertebrate hedgehog signaling pathway have been identified (Ingham, 1998). In particular, two zinc finger transcription factors, Gli1 and Gli2, have been proposed as intermediaries in Shh signaling (Ruiz i Altaba, 1999). One view of the initial steps in Shh signal transduction argues that the level of Gli activity varies in proportion to the concentration of extracellular Shh (Ingham, 1998), and thus different levels of Gli activity may repress or activate different class I and class II homeobox genes. However, ventral neuronal pattern is surprisingly normal in mice containing mutations in both the *Gli1* and *Gli2* genes (Ding et al., 1998; Matise et al., 1998). These findings raise the possibility (see Krishnan et al., 1997; Lewis et al., 1999) that additional transcriptional mediators participate in the initial interpretation of graded Shh signals within ventral progenitor cells.

The uncertainty that persists about the initial stages of Shh signal transduction in neural cells also leaves unresolved the issue of whether Shh acts independently to repress class I and to activate class II genes. The pairs of class I and class II proteins that form complementary domain boundaries are potent repressors of each other's expression. Thus, the repression of class I genes by Shh could depend on the activation of class II gene expression. Alternatively, the requirement for class II protein expression on Shh signaling may depend on the Shh repression of class I protein expression. A similar derepression mechanism has been suggested to operate

during *Drosophila* development, in the dpp-mediated patterning of imaginal disc cells (Campbell and Tomlinson, 1999; Jazwinska et al., 1999; Minami et al., 1999).

The cross-regulatory interactions revealed for class I and class II proteins also have implications for the lineage relationship of neurons generated in the ventral neural tube. Lineage tracing studies have reported a temporal change in the extent to which clonally-related cells disperse along the dorsoventral axis of the ventral neural tube (Leber and Sanes, 1995). After early stage marking of ventral progenitors, clonally-related progeny spread widely along the dorsoventral axis of the ventral neural tube and acquire different neuronal identities (Leber and Sanes, 1995; Erskine et al., 1998). But, the progeny of clones marked at later developmental stages are restricted to narrower dorsoventral domains, and within these domains cells acquire more uniform neuronal fates (Leber and Sanes, 1995). The timing of the cross-regulatory interactions between class I and class II proteins that seem to confer progenitor domain identity matches well with the time of restriction in clonal cell dispersal, suggesting a causal relationship between these two processes. The homeodomain proteins that define an individual ventral progenitor domain could control the surface properties of progenitor cells and restrict their intermixing along the dorsoventral axis, in a manner analogous with mechanisms that establish segmental domains along the rostrocaudal axis of the hindbrain (Lumsden and Krumlauf, 1996; Xu et al., 1999).

Control of Neuronal Identity by a Homeodomain Protein Code

This study has relied on ectopic expression methods to address the roles of Nkx6.1, Nkx2.2 and Irx3 in specifying the fate of V2 neurons, MNs and V3 neurons. The results herein show that Nkx2.2 activity is sufficient to induce V3 neurons, that Nkx6.1 activity in the absence of Irx3 induces MNs, whereas Nkx6.1 activity in the presence of Irx3 induces V2 neurons. The inferences derived from these gain-of-function studies are supported by the switches in neuronal fate that occur in mice in which individual class I and class II proteins have been inactivated by gene targeting. In mice lacking Pax6 activity, the dorsal expansion in the domain of Nkx2.2 expression is accompanied by an expansion in the domain of V3 neuron generation, and by the loss of MNs (Ericson et al., 1997a). Conversely, the loss of Nkx2.2 results in the loss of V3 neurons and in the ectopic generation of MNs within the p3 domain (Briscoe et al., 1999). In addition, the loss of Nkx6.1 activity depletes the ventral neural tube of many MNs and V2 neurons (Sander et al., submitted).

How do class I and class II proteins control neuronal subtype identity? The final cell division of certain ventral progenitors is accompanied by the onset of expression of a distinct set of homeodomain proteins, notably MNR2 and Lim3 (Tanabe et al., 1995; Ericson et al., 1997; Sharma et al., 1998). Ectopic expression of MNR2 is able to induce MN differentiation independent of dorsoventral position, and ectopic expression of Lim3

induces V2 neurons (Tanabe et al., 1998). The studies herein indicate that class I and class II proteins function upstream of MNR2 and Lim3. Thus within the pMN and p2 domains, the actions of progenitor homeodomain proteins in specifying neuronal subtype identity are likely to be mediated through MNR2 and Lim3. Subtype determinant factors with equivalent functions may therefore be expressed by cells in the other ventral progenitor domains.

These findings provide further support for the idea that the activity of individual homeodomain proteins can direct specific neuronal fates in the developing spinal cord. It is shown here that Nkx2.2 can specify V3 neuronal identity. In previous studies MNR2 has been shown to specify MN identity and Lim3 to direct V2 neuronal identity (Tanabe et al., 1998). Thus, the fate of other classes of neurons in the ventral spinal cord, and perhaps in other regions of the vertebrate central nervous system, may be controlled through the actions of similarly dedicated transcription factors. The activities of Nkx6.1 revealed in these studies also provide a further insight into the hierarchical functions of homeodomain proteins in specifying spinal MN identity. Nkx6.1 can induce the expression of both MNR2 and Lim3 in MN progenitors, and like MNR2, is able to specify MN fate in dorsal neural tube cells. Thus, it seems possible that Nkx6.1 functions upstream of MNR2 in a linear pathway of MN generation in the chick embryo.

Linking Graded Extracellular Signals to Neuronal Subtype

Diversity

A set of seven homeodomain proteins defines five neural progenitor domains with a fundamental role in the organization of ventral neural pattern. The analysis of these homeodomain proteins suggests that ventral patterning proceeds in three stages: (1) the regulation of class I and class II proteins by graded Shh signals, (2) the refinement and maintenance of progenitor domain identity by cross-repressive interactions between homeodomain proteins, and (3) the translation of a homeodomain protein code into neuronal subtype identity. The central features of this model may apply to other vertebrate tissues in which cell pattern is regulated by local sources of extrinsic signals. Consistent with this idea, cross-regulatory interactions between transcription factors have been suggested to refine cell pattern in the embryonic mesoderm and in the pituitary gland (Papin and Smith, 2000; Dasen and Rosenfeld, 1999)

Finally, it is noted that the principles of the model of ventral patterning outlined here resemble those involved in subdividing the *Drosophila* embryo (Lawrence, 1992). Graded Shh signaling subdivides the ventral neural tube into five domains, just as graded levels of the dorsal protein establish five distinct regions of the early *Drosophila* embryo (Huang et al., 1997), suggesting an upper limit to the number of distinct cell fates that can be generated in response to a single gradient signaling system. In addition, the graded anterioposterior distribution of maternally-supplied factors in the *Drosophila* embryo is known to initiate the expression of

a set of proteins encoded by the *gap* genes (Struhl et al., 1992). Subsequent cross-regulatory interactions establish and maintain sharp boundaries in the expression of gap proteins, and their activities within individual domains control later aspects of cell pattern (Kraut and Levine, 1991; Wu et al., 1998). Thus in the neural tube and the *Drosophila* embryo, the cross-repression of genes whose initial expression is controlled by graded upstream signals provides an effective mechanism for establishing and maintaining progenitor domains and for imposing cell type identity.

REFERENCES FOR FIRST SERIES OF EXPERIMENTS

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T. M., and Sockanathan, S. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. Neuron *23*, 659-674.

Briscoe, J., Sussel, L., Serup, P., Hartigan-O'Connor, D., Jessell, T. M., Rubenstein, J. L., and Ericson, J. (1999). Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature *398*, 622-622.

Campbell, G., and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of Drosophila: regulation of Dpp targets by brinker. Cell *96*, 553-562.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H., and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature *383*, 407-413.

Dasen, J. S., and Rosenfeld, M. G. (1999). Combinatorial codes in signaling and synergy: lessons from pituitary development. Curr. Opin. Genet. Dev. *9*, 566-574.

Ding, Q., Motoyama, J., Gasca, S., Mo, R., Sasaki, H., Rossant, J., and Hui, C. C. (1998). Diminished Sonic hedgehog signaling and lack of floor plate differentiation in Gli2 mutant mice. Development *125*, 2533-2543.

Ericson, J., Morton, S., Kawakami, A., Roelink, H., and Jessell, T. M. (1996). Two critical periods of Sonic Hedgehog signaling required for the specification of motor neuron identity. Cell *87*, 661-673.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M., and Briscoe, J. (1997a). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. Cell *90*, 169-180.

Ericson, J., Briscoe, J., Rashbass, P., van Heyningen, V., and Jessell, T. M. (1997b). Graded sonic hedgehog signaling and the specification of cell fate in the ventral neural tube. Cold Spring Harb. Symp. Quant. Biol.

62, 451-466.

Erskine, L., Patel, K., and Clarke, J. D. (1998). Progenitor dispersal and the origin of early neuronal phenotypes in the chick embryo spinal cord. Dev. Biol. *199*, 26-41.

Funayama, N., Sato, Y., Matsumoto, K., Ogura, T., and Takahashi, Y. (1999). Coelom formation: binary decision of the lateral plate mesoderm is controlled by the ectoderm. Development *126*, 4129-4138.

Goulding M.D., Lumsden A., and Gruss P. (1993). Signals from the notochord and floor plate regulate the region-specific expression of two Pax genes in the developing spinal cord. Development *117*, 1001-1016

Hamburger, V., and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. J. Morphol. 88, 49-92.

Huang, A. M., Rusch, J., and Levine, M. (1997). An anteroposterior Dorsal gradient in the Drosophila embryo. Genes Dev. *11*, 1963-1973.

Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J., and Sutrave, P. (1987). Adaptor plasmids simplify the insertion of foreign DNA into helper- independent retroviral vectors. J. Virol. *61*, 3004-3012.

Ingham, P. W. (1998). Transducing Hedgehog: the story so far. EMBO J. *17*, 3505-3511.

Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S., and Rushlow, C. (1999). The Drosophila gene brinker reveals a novel mechanism of Dpp target gene regulation. Cell *96*, 563-573.

Kraut, R., and Levine, M. (1991). Spatial regulation of the gap gene giant during Drosophila development. Development *111*, 601-609.

Krishnan, V., Pereira, F. A., Qiu, Y., Chen, C. H., Beachy, P. A., Tsai, S. Y., and Tsai, M. J. (1997). Mediation of Sonic hedgehog-induced expression of COUP-TFII by a protein phosphatase. Science *278*, 1947-1950.

Langman, J., Guerrant, R.L., and Freeman, G. G. (1966). Behavior of neuroepithelial cells during closure of neural tube. J. Comp. Neurol. *127*, 399-411.

Lawrence, P. (1992). The Making of a Fly. Oxford, Blackwell Scientific.

Lawrence, P. A., and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? Cell *85*, 951-961.

Leber, S. M., and Sanes, J. R. (1995). Migratory paths of neurons and glia in the embryonic chick spinal cord. J Neurosci. *15*, 1236-1248.

Lewis, K. E., Drossopoulou, G., Paton, I. R., Morrice, D. R., Robertson, K. E., Burt, D. W., Ingham, P. W., and Tickle, C. (1999). Expression of ptc and gli genes in talpid3 suggests bifurcation in Shh pathway. Development *126*, 2397-2407.

Lumsden, A., and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. Science *274*, 1109-1115.

Mansouri, A., and Gruss, P. (1998). Pax3 and Pax7 are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord. Mech. Dev. *78*, 171-178.

Marti, E., Takada, R., Bumcrot, D. A., Sasaki, H., and McMahon, A. P. (1995). Distribution of Sonic hedgehog peptides in the developing chick and mouse embryo. Development *121*, 2537-2547.

Matise, M. P., Epstein, D. J., Park, H. L., Platt, K. A., and Joyner, A. L. (1998). Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development *125*, 2759-2770.

McDowell, N., and Gurdon, J. B. (1999). Activin as a morphogen in Xenopus mesoderm induction. Semin. Cell Dev. Biol. *10*, 311-317.

Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H., and Tabata, T. (1999). Brinker is a target of Dpp in

Drosophila that negatively regulates Dpp- dependent genes. Nature *398*, 242-246.

Morgan, B. A., and Fekete, D. M. (1996). Manipulating gene expression with replication-competent retroviruses. Methods Cell Biol. *51*, 185-218.

Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. Biochem. Biophys. Res. Commun. *230*, 376-380.

Pabst, O., Herbrand, H., and Arnold, H. H. (1998). Nkx2.9 is a novel homeobox transcription factor which demarcates ventral domains in the developing mouse CNS. Mech. Dev. *73*, 85-93.

Papin, C., and Smith, J. C. (2000). Gradual refinement of activin-induced thresholds requires protein synthesis. Dev. Biol. *217*, 166-172.

Pierani, A., Brenner-Morton, S., Chiang, C., and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. Cell *97*, 903-915.

Qiu, M., Shimamura, K., Sussel, L., Chen, S., and Rubenstein, J. L. (1998). Control of anteroposterior and dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. Mech. Dev. *72*, 77-88.

Roelink, H., Porter, J. A., Chiang, C., Tanabe, Y., Chang, D. T., Beachy, P. A., and Jessell, T. M. (1995). Floor plate and motor neuron induction by different concentrations of the amino-terminal cleavage product of sonic hedgehog autoproteolysis. Cell *81*, 445-455.

Rowitch, D. H., S.-Jacques. B., Lee, S. M., Flax, J. D., Snyder, E. Y., and McMahon, A. P. (1999). Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. J. Neurosci. *19*, 8954-8965.

Ruiz i Altaba, A. (1999). Gli proteins and Hedgehog signaling: development and cancer. Trends Genet. *15*, 418-425.

Sander, M., Paydar, S., Ericson, J., Briscoe, J., German, M., Jessell, T. M., and Rubenstein, J.L.R. (2000). Ventral Neural Tube Patterning by Nkx Homeobox Genes: Nkx6.1 Controls Somatic Motor Neuron and Ventral Interneuron Fates. Submitted.

Schaeren-Wiemers, N., and Gerfin-Moser, A. (1993). A single protocol to detect transcripts of various types and expression levels in neural tissue and cultured cells: in situ hybridization using digoxigenin-labelled cRNA probes. Histochemistry *100*, 431-440.

Sharma, K., Sheng, H. Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., and Pfaff, S. L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell *95*, 817-828.

Smith, J. C. (1995). Mesoderm-inducing factors and mesodermal patterning. Curr. Opin. Cell Biol. 7, 856-861.

Struhl, G., Johnston, P., and Lawrence, P. A. (1992). Control of Drosophila body pattern by the hunchback morphogen gradient. Cell *69*, 237-249

Tanabe, Y., William, C., and Jessell, T. M. (1998). Specification of motor neuron identity by the MNR2 homeodomain protein. Cell *95*, 67-80.

Wolpert, L. (1969). Positional information and the spatial pattern of cellular differentiation. J. Theor. Biol. *25*, 1-47.

Wu, X., Vakami, R., and Small, S. (1998). Two distinct mechanisms for differential positioning of gene expression borders involving the Drosophila gap protein giant. Development *125*, 3765-3774.

Xu, Q., Mellitzer, G., Robinson, V., and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. Nature *399*, 267-271.

Yamada, T., Pfaff, S. L., Edlund, T., and Jessell, T. M. (1993). Control of cell pattern in the neural tube: motor neuron induction by diffusible factors from notochord and floor plate. Cell *73*, 673-686.

Second Series of Experiments

During the development of the embryonic central nervous system (CNS) the mechanisms that specify regional identity and neuronal fate are intimately linked (1,2). In the ventral half of the CNS, for example, the secreted factor Sonic hedgehog (Shh) has a fundamental role in controlling both regional pattern and neuronal fate (3). The genetic programs activated in neural progenitor cells in response to Shh signaling, however, remain poorly defined. Emerging evidence suggests that homeobox genes function as critical intermediaries in the neural response to Shh signals (1-3). In particular, genetic studies in mice have shown that two Shh-regulated homeobox genes, *Nkx2.1* and *Nkx2.2*, control dorsoventral fates both in the basal telencephalon and in the ventral-most regions of the spinal cord (4, 5). These findings raise the possibility that members of the *Nkx* class of homeobox genes have a central role in imposing regional pattern and neuronal fate in the ventral region of the CNS.

A recently identified *Nkx* gene, *Nkx6.1*, is expressed by neural progenitor cells throughout the ventral third of the neural tube (5-7), suggesting that it may have a pervasive role in ventral neural patterning. To define the role of *Nkx6.1* in neural development, patterns of neurogenesis were compared in the embryonic spinal cord and hindbrain of wild type mice and mice lacking *Nkx6.1* (8). In wild type embryos, neural expression of *Nkx6.1* is first detected at spinal cord and caudal hindbrain levels

at ~e8.5 (data not shown; ref 6) and by e9.5 the gene is expressed throughout the ventral third of the neural tube (Figure 9A). The expression of *Nkx6.1* persists until at least e12.5 (Figures 9B, 9C; and data not shown). *Nkx6.1* expression was also detected in mesodermal cells flanking the ventral spinal cord (Figures 9B, 9C). To define more precisely the domain of expression of Nkx6.1 compared was Nkx6.1 expression with that of nine homeobox genes - *Pax3, Pax7, Gsh1, Gsh2, Pax6, Dbx1, Dbx1, Dbx2 and Nkx2.9* - that have been shown to define discrete progenitor cell domains along the dorsoventral axis of the ventral neural tube (9-14).

This analysis revealed that the dorsal boundary of *Nkx6.1* expression is positioned ventral to the boundaries of four genes expressed by dorsal progenitor cells: *Pax3*, *Pax7*, *Gsh1* and *Gsh2* (Figures 9I, 9N; and data not shown). Within the ventral neural tube, the dorsal boundary of *Nkx6.1* expression is positioned ventral to the domain of *Dbx1* expression and close to the ventral boundary of *Dbx2* expression (Figures 9G, 9H, and 9P). The domain of Pax6 expression extends ventrally into the domain of *Nkx6.1* expression (Figure 9O), whereas the expression of *Nkx2.2* and *Nkx2.9* overlaps with the ventral-most domain of *Nkx6.1* expression (Figures 9O, 9Q).

To address the function of *Nkx6.1* in neural development, progenitor cell identity and the pattern of neuronal differentiation in *Nkx6.1* null mutant mice was analyzed (8). Detected was a striking change in the profile of expression of three homeobox genes, *Dbx2*, *Gsh1* and *Gsh2*,

in *Nkx6.1* mutants. The domains of expression of *Dbx2*, *Gsh1* and *Gsh2* each expanded into the ventral neural tube (Figures 9K-9M; and data not shown). At e10.5, *Dbx2* was expressed at high levels by progenitor cells adjacent to the floor plate, but at this stage ectopic *Dbx2* expression was detected only at low levels in regions of the neural tube that generate motor neurons (Figure 9K). By e12.5, however, the ectopic ventral expression of *Dbx2* had become more uniform, and now clearly included the region of motor neuron and V2 neuron generation (Figure 9L). Similarly, in *Nkx6.1* mutants, both *Gsh1* and *Gsh2* were ectopically expressed in a ventral domain of the neural tube, and also in adjacent paraxial mesodermal cells (Figure 9M; data not shown).

The ventral limit of Pax6 expression was unaltered in *Nkx6.1* mutants, although the most ventrally located cells within this progenitor domain expressed a higher level of Pax6 protein than in wild type embryos (Figures 9O, 9S). No change was detected in the patterns of expression of *Pax3*, *Pax7*, *Dbx1*, *Nkx2.2* or *Nkx2.9* in *Nkx6.1* mutant embryos (Figures 9R-9U; and data not shown). Importantly, the level of Shh expression by floor plate cells was unaltered in *Nkx6.1* mutants (Figures 9N and 9R). Thus, the loss of *Nkx6.1* function deregulates the patterns of expression of a selected subset of homeobox genes in ventral progenitor cells, without an obvious effect on Shh levels (Figures 9D, 9E). The role of Shh in excluding *Dbx2* from the most ventral region of the neural tube (11) appears therefore to be mediated through the induction of Nkx6.1 expression. Consistent with this view, ectopic

expression of Nkx6.1 represses *Dbx2* expression in chick neural tube (12). The detection of sites of ectopic *Gsh1/2* expression in the ventral neural tube as well as the paraxial mesoderm, both sites of *Nkx6.1* expression, suggests that *Nkx6.1* has a general role in restricting *Gsh1/2* expression. The signals that promote ventral *Gsh1/2* expression in *Nkx6.1* mutants remain unclear, but could involve factors other than Shh that are secreted by the notochord (15).

The domain of expression of *Nkx6.1* within the ventral neural tube of wild type embryos encompasses the progenitors of three main neuronal classes: V2 interneurons, motor neurons and V3 interneurons (5, 6, 10-13) (Figures 10A-10D). It was examined whether the generation of any of these neuronal classes is impaired in *Nkx6.1* mutants, focusing first on the generation of motor neurons. In *Nkx6.1* mutant embryos there was a marked reduction in the number of spinal motor neurons, as assessed by expression of the homeodomain proteins Lhx3, Isl1/2 and HB9 (16, 17) (Figures 10E-10L), and by expression of the gene encoding the transmitter synthetic enzyme choline acetyltransferase (data not shown). In addition, few if any axons were observed emerging from the ventral spinal cord (data not shown). The incidence of motor neuron loss, however, varied along the rostrocaudal axis of the spinal cord. Few if any motor neurons were detected at caudal cervical and upper thoracic levels of *Nkx6.1* mutants analyzed at e11-e12.5 (Figures 10M, 10N, 10Q, 10R), whereas motor neuron number was reduced only by 50-75% at more caudal levels (Figures

10O, 10P, 10S, 10T; and data not shown). At all axial levels, the initial reduction in motor neuron number persisted at both e12.5 and p0 (Figures 10M-10T and data not shown), indicating that the loss of Nkx6.1 activity does not simply delay motor neuron generation. Moreover, no increase was detected in the incidence of TUNEL$^+$ cells in *Nkx6.1* mutants (data not shown), indicating that the depletion of motor neurons is not the result of apoptotic death.

The persistence of some spinal motor neurons in *Nkx6.1* mutants raised the possibility that the generation of particular subclasses of motor neurons is selectively impaired. To address this issue, the expression of markers of distinct subtypes of motor neurons at both spinal and hindbrain levels of *Nkx6.1* mutant embryos was monitored. At spinal levels, the extent of the reduction in the generation of motor neurons that populate the median (MMC) and lateral (LMC) motor columns was similar in *Nkx6.1* mutants, as assessed by the number of motor neurons that coexpressed Isl1/2 and Lhx3 (defining MMC neurons, refs 16, 17) (Figures 11A, 11B) and by the expression of *Raldh2* (defining LMC neurons, refs.17, 18) (Figures 11C, 11D). In addition, the generation of autonomic visceral motor neurons was reduced to an extent similar to that of somatic motor neurons at thoracic levels of the spinal cord of e12.5 embryos (data not shown). Thus, the loss of *Nkx6.1* activity depletes the major subclasses of spinal motor neurons to a similar extent.

At hindbrain levels, *Nkx6.1* is expressed by the progenitors of both somatic and visceral motor neurons (Figures 11E, 11F; and data not shown). Therefore, it was examined whether the loss of *Nkx6.1* might selectively affect subsets of cranial motor neurons. Detected was a virtually complete loss in the generation of somatic motor neurons (hypoglossal and abducens) in *Nkx6.1* mutants, as assessed by the absence of dorsally generated HB9$^+$ motor neurons (Figures 11G, 11H; and data not shown, refs 5, 17). In contrast, there was no change in the initial generation of any of the cranial visceral motor neuron populations, assessed by coexpression of Isl1 and Phox2a (5, 19) within ventrally generated motor neurons (Figures 11I, 11J; and data not shown). Moroever, at rostral cervical levels, the generation of spinal accessory motor neurons (10) was also preserved in *Nkx6.1* mutants (data not shown). Thus, in the hindbrain the loss of *Nkx6.1* activity selectively eliminates the generation of somatic motor neurons, while leaving visceral motor neurons intact. Cranial visceral motor neurons, unlike spinal visceral motor neurons, derive from progenitors that express the related Nkx genes *Nkx2.2* and *Nkx2.9* (5). The preservation of cranial visceral motor neurons in *Nkx6.1* mutant embryos may therefore reflect the dominant activities of *Nkx2.2* and *Nkx2.9* within these progenitor cells.

Next examined was whether the generation of ventral interneurons is affected by the loss of *Nkx6.1* activity. V2 and V3 interneurons are defined, respectively, by expression of Chx10 and *Sim1* (5,17) (Figures 12A, 12G).

A severe loss of Chx10 V2 neurons was detected in *Nkx6.1* mutants at spinal cord levels (Figure 12B), although at hindbrain levels of *Nkx6.1* mutants ~50% of V2 neurons persisted (data not shown). In contrast, there was no change in the generation of *Sim1* V3 interneurons at any axial level of *Nkx6.1* mutants (Figure 12H). Thus, the elimination of *Nkx6.1* activity affects the generation of only one of the two major classes of ventral interneurons that derive from the Nkx6.1 progenitor cell domain.

Evx1$^+$, Pax2$^+$ V1 interneurons derive from progenitor cells located dorsal to the Nkx6.1 progenitor domain, (Figure 12B) within a domain that expresses *Dbx2*, but not *Dbx1* (11, 20, 21). Since *Dbx2* expression undergoes a marked ventral expansion in *Nkx6.1* mutants, it was examined whether there might be a corresponding expansion in the domain of generation of V1 neurons. In *Nkx6.1* mutants, the region that normally gives rise to V2 neurons and motor neurons now also generated V1 neurons, as assessed by the ventral shift in expression of the En1 and Pax2 homeodomain proteins (Figures 12B, 12C, 12E, 12F). Consistent with this, there was a 2-3 fold increase in the total number of V1 neurons generated in *Nkx6.1* mutants (Figures 12C, 12D). In contrast, the domain of generation of Evx1/2 V0 neurons, which derive from the *Dbx1* progenitor domain (11), was unchanged in *Nkx6.1* mutants (Figures 12I, 12J). Thus, the ventral expansion in *Dbx2* expression is accompanied by a selective switch in interneuronal fates, from V2 neurons to V1 neurons. In addition, it was observed that some neurons within the ventral spinal cord of *Nkx6.1* mutants coexpressed the V1

marker En1 and the V2 marker Lhx3 (Figures 12K, 12L). The coexpression of these markers is rarely if ever observed in single neurons in wild type embryos (22). Thus, within individual neurons in *Nkx6.1* mutants, the ectopic program of V1 neurogenesis appears to be initiated in parallel with a residual, albeit transient, program of V2 neuron generation. This result complements observations in *Hb9* mutant mice, in which the programs of V2 neuron and motor neuron generation coincide transiently within individual neurons (17, 23).

Taken together, the findings herein reveal an essential role for the *Nkx6.1* homeobox gene in the specification of regional pattern and neuronal fate in the ventral half of the mammalian CNS. Within the broad ventral domain within which *Nkx6.1* is expressed (Figure 13A), its activity is required to promote motor neuron and V2 interneuron generation and to restrict the generation of V1 interneurons (Figure 13B). The loss of motor neurons and V2 neurons could be a direct consequence of the loss of *Nkx6.1* activity, since the depletion of these two neuronal subtypes is evident at stages when only low levels of *Dbx2* are expressed ectopically in most regions of the ventral neural tube. Consistent with this view, the ectopic expression of *Nkx6.1* is able to induce both motor neurons and V2 neurons in chick neural tube (12). V3 interneurons and cranial visceral motor neurons derive from a set of *Nkx6.1* progenitors that also express Nkx2.2 and Nkx2.9 (5) (Figure 13A). The generation of these two neuronal subtypes is unaffected by the loss of *Nkx6.1* activity, suggesting that the actions of *Nkx2.2* and

Nkx2.9 dominate over that of Nkx6.1 within these progenitors. The persistence of some spinal motor neurons and V2 neurons in *Nkx6.1* mutants could reflect the existence of a functional homologue within the caudal neural tube.

The role of *Nkx6.1* revealed in these studies, taken together with previous findings (4, 5), suggests a model in which the spatially restricted expression of *Nkx* genes within the ventral neural tube (Figure 13) has a pivotal role in defining the identity of ventral cell types induced in response to graded Shh signaling. Strikingly, in *Drosophila*, the *Nkx* gene *NK2* has been shown to have an equivalent role in specifying neuronal fates in the ventral nerve cord (24). Moreover, the ability of *Nkx6.1* to function as a repressor of the dorsally expressed *Gsh1/2* homeobox genes parallels the ability of *Drosophila NK2* to repress *Ind*, a *Gsh1/2*-like homeobox gene (25). Thus, the evolutionary origin of regional pattern along the dorsoventral axis of the central nervous system may predate the divergence of invertebrate and vertebrate organisms.

REFERENCES AND NOTES FOR SECOND SERIES OF EXPERIMENTS

20. J.L. Rubenstein et al., *Annu Rev Neurosci.* **21**, 45-77 (1998); S.A. Anderson, D.D. Eisenstat, L. Shi, J.L. Rubenstein, *Science* **278**, 474-476 (1997)

2. A. Lumsden , R. Krumlauf, *Science* **274**, 1109-15 (1996).

3. Y. Tanabe, T.M. Jessell, *Science* **274**, 1115-23 (1996); M. Hammerschmidt, A. Brook, A.P. McMahon *Trends Genet.* **13**, 14-21 (1997); J. Ericson, et al., *Cold Spring Harb. Symp. Quant. Biol.* **62**, 451-66 (1997).

4. L. Sussel, O. Marin, S. Kimura, J.L. Rubenstein, *Development* **126**, 3359-70 (1999).

5. J. Briscoe, et al., *Nature* **398**, 622-7 (1999).

6. M. Qiu, K. Shimamura, L. Sussel, S. Chen, J. L. Rubenstein, *Mech. Dev.* **72**, 77-88 (1998).

7. O. Pabst, H. Herbrand, H. H. Arnold, *Mech. Dev.* **73**, 85-93 (1998).

8. M. Sander et al., submitted (2000). A null mutation in Nkx6.1 was generated by gene targeting in 129-strain ES cells. Briefly, a 800 bp NotI fragment containing part of exon1 was excised and replaced by a PGK-neo cassette. Mice were genotyped by Southern blotting of EcoRI digested genomic DNA with a 3' external probe to the targeting construct. The size of the wild-type allele is 10 kb, and of the targeted allele 4.3 kb. The mice were outcrossed with C57Bl6. Heterozygotes appeared normal, whereas homozygous mutant embryos (which are born at Mendelian frequency) died soon after birth. Newborn mutants were slightly smaller (~85% normal weight), exhibited a persistently flexed body posture and lacked spontaneous movements. Tactile stimulation elicited weak movements of their extremities but no movement of their trunk was detected.

9. Localization of mRNA was performed by in situ hybridization following the method of Schaeren-

Wiemers and Gerfin-Moser; Histochemistry *100*, 431 (1993). The *Dbx2* riboprobe comprised the 5' EcoR1 fragment of the mouse cDNA; (11). Probes for other cDNAs were used as described: *Nkx2.9* (5), *Nkx6.1* (6), *Dbx1* (11), *Gsh1* (14), *Gsh2* (14), *Pax3* (13), Chx10 (10), Sim1 (5), En1 (11, 20, 21), Evx1 (11, 21) and RALDH2 (18). Protein expression was localized by indirect fluorescence immunocytochemistry or peroxidase immunohistochemistry (3, 5). Nkx6.1 was detected with a rabbit antiserum (5). Antisera against Shh, Pax7, Isl1/2, HB9, Lhx3, Chx10, Phox2a/b, En1, Pax2 have been described (5, 10). Fluorescence detection was carried out using an MRC 1024 Confocal Microscope.

10. J. Ericson, et al., Cell. **90**, 169-80 (1997).

11. A. Pierani, S. Brenner-Morton, C. Chiang, T. M. Jessell, *Cell* **97**, 903-15 (1999).

12. J. Briscoe et al., submitted (2000).

13. M.D. Goulding et al., *EMBO J.* **10**, 1135-47 (1991).

14. M. T. Valerius, H. Li, J.L. Stock, M. Weinstein, S. Kaur, G. Singh, S.S. Potter, Dev. Dyn. **203**, 337-51 (1995).

15. M. Hebrok, S.K. Kim, D.A. Melton, *Genes Dev.* **12**, 1705-13 (1998).

16. T. Tsuchida, et al., *Cell* **79**, 957-70 (1994).

17. S. Arber, B. Han, M. Mendelsohn, M. Smith, T.M. Jessell, S. Sockanathan, *Neuron* ***23***, 659-74 (1999).

18. S. Sockanathan, T.M. Jessell, *Cell* **94**, 503-14 (1998).

19. A. Pattyn, X. Morin, H. Cremer, C. Goridis, J.F. Brunet, *Development* ***124***, 4065-75 (1997).

20. M.P. Matise, A.L. Joyner, *J. Neurosci.* **17**, 7805-16 (1998).

21. J.D. Burrill, L. Moran, M.D. Goulding, H.

Saueressig, *Development* **124**, 4493-503 (1997).

22. J. Ericson et al., *Cell* **87**, 661-73 (1996).

23. J. Thaler et al., *Neuron* **23**, 675-87 (1999).

24. J. A. McDonald, S. Holbrook, T. Isshiki, J. Weiss, C.Q. Doe, D. M. Mellerick. *Genes Dev.* **12**, 3603-12 (1998). H. Chu; C. Parras; K. White; F. Jimenez, *Genes and Development*. **12**, 3613-24 (1998).

25. J. B. Weiss, T. Von Ohlen, D.M. Mellerick, G. Dressler, C.Q. Doe, M.P.Scott, *Genes Dev.* **12**,3591-602 (1998).

26. We thank the following people for cDNAs: P. Gruss (*Pax3*), S. Potter (*Gsh1&2*), F.Ruddle (*Dbx1*), R. McInnes (*Chx10*), A. Joyner (*En1*), G. Martin (*Evx1*), M.Tessier-Lavigne (*Sim1*), C. Gall (*ChAT*); and the following people for antibodies: J.F.Brunet (anti-Phox2), H. Westphal (Lhx3).

What is claimed is:

1. A genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein.

2. The genetically engineered cell of claim 1, wherein the neural stem cell is a mammalian neural stem cell.

3. The genetically engineered cell of claim 2, wherein the mammalian stem cell is a human neural stem cell.

4. A method of generating a genetically engineered motor neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein which comprises treating the genetically engineered cell of claim 1 under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein so as to thereby generate the genetically engineered motor neuron.

5. The method of claim 4, wherein the neural stem cell is a mammalian cell neural stem cell.

6. The method of claim 5, wherein the mammalian neural stem cell is a human neural stem cell.

7. A genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein.

8. The genetically engineered stem cell of claim 7, wherein the neural stem cell is a mammalian neural stem cell.

9. The genetically engineered cell of claim 8, wherein the mammalian neural stem cell is a human neural stem cell.

10. A method of generating a genetically engineered V2 neuron which is capable of expressing homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein which comprises treating the genetically engineered cell of claim 7 under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx6.1 protein and homeodomain transcription factor Irx3 protein so as to thereby generate the genetically engineered V2 neuron.

11. The method of claim 10, wherein the neural stem cell is a mammalian neural stem cell.

12. The method of claim 11, wherein the mammalian neural stem cell is a human neural stem cell.

13. A genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein.

14. The neural stem cell of claim 13, wherein the neural stem cell is a mammalian neural stem cell.

15. The neural stem cell of claim 14, wherein the mammalian neural stem cell is a human neural stem cell.

16. A method of generating a genetically engineered V3 neuron which is capable of expressing homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein which comprises treating the genetically engineered cell of claim 13 under conditions such that the retroviral expression system expresses homeodomain transcription factor Nkx2.2 protein or homeodomain transcription factor Nkx2.9 protein so as to thereby generate the genetically engineered V3 neuron.

17. The method of claim 16, wherein the neural stem cell is a mammalian neural stem cell.

18. The method of claim 17, wherein the mammalian neural stem cell is a human neural stem cell.

19. A method of treating subject having a motor neuron injury or a motor neuron disease comprising:
implanting in injured or diseased neural tissue of the subject a genetically engineered cell comprising a neural stem cell and retroviral expression system in the neural stem cell, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 pr otein.

20. The method of claim 19, wherein the neural stem cells are transfected with the retroviral expression system in vitro and implanted into the subject.

21. The method of claim 19, wherein the motor neuron disease is amyotrophic lateral sclerosis, spinal muscular atrophy or any motor neuron degenerative disease.

22. The method of claim 19, wherein the neural stem cells are from the developing mammalian nervous system.

23. The method of claim 19, wherein the neural stem cells are from the adult mammalian nervous system.

24. A method of treating subject having a motor neuron

injury or a motor neuron disease comprising: administering to injured or diseased neural tissue of adult spinal cord a retroviral expression system, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein.

25. The method of claim 24, wherein the motor neuron injury is a spinal cord injury.

26. The method of claim 24, wherein the motor neuron disease is amyotrophic lateral sclerosis, spinal muscular atrophy or any motor neuron degenerative disease.

27. A method of treating subject having a motor neuron injury or a motor neuron disease comprising:

a) transfecting neural stem cells with a retroviral vector, which is capable of expressing homeodomain transcription factor Nkx6.1 protein but does not express homeodomain transcription factor Irx3 protein or homeodomain transcription factor Nkx2.2 protein; and

b) injecting the transfected neural stem cells of step (a) into the central canal of the spinal cord under conditions which allow the injected transfected neural stem cells to be incorporated into the ependimal layer of the

spinal cord.

28. The method of claim 27, wherein the neural stem cells are from the developing mammalian nervous system.

29. The method of claim 27, wherein the neural stem cells are from the adult mammalian nervous system.

30. A method of determining whether a chemical compound affects the generation of a motor neuron from a neural stem cell which comprises:

b) contacting the genetically engineered cell of claim 1 with the chemical compound under conditions such that in the absence of the compound the neural stem cell expresses homeodomain transcription factor Nkx6.1 protein and generates a motor neuron; and

c) determining what effect, if any, the compound has on generation of the motor neuron.

31. The method of claim 30, wherein the chemical compound promotes generation of the motor neuron.

FIGURE 1A

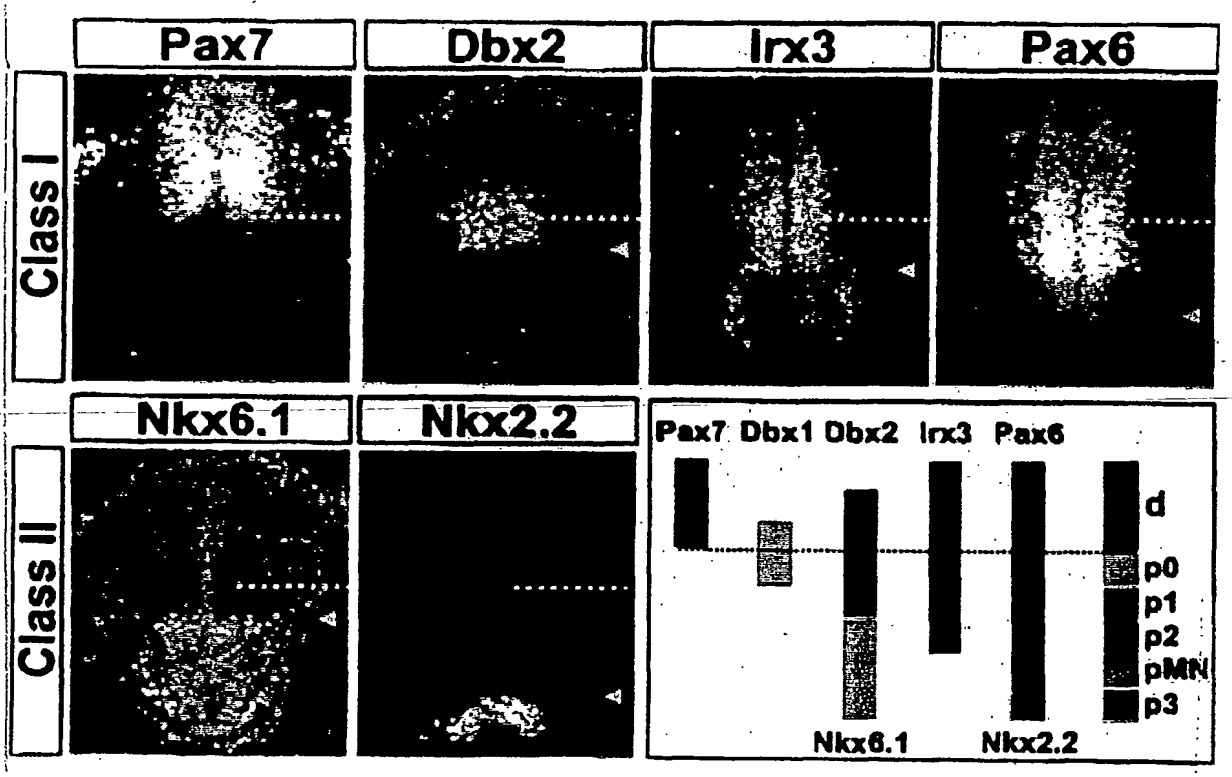

Pax7
Dbx2
Irx3
Pax6
Class I
Nkx6.1
Nkx2.2
Class II
Pax7 Dbx1 Dbx2 Irx3 Pax6
d
p0
p1
p2
pMN
p3
Nkx6.1
Nkx2.2

FIGURE 1B

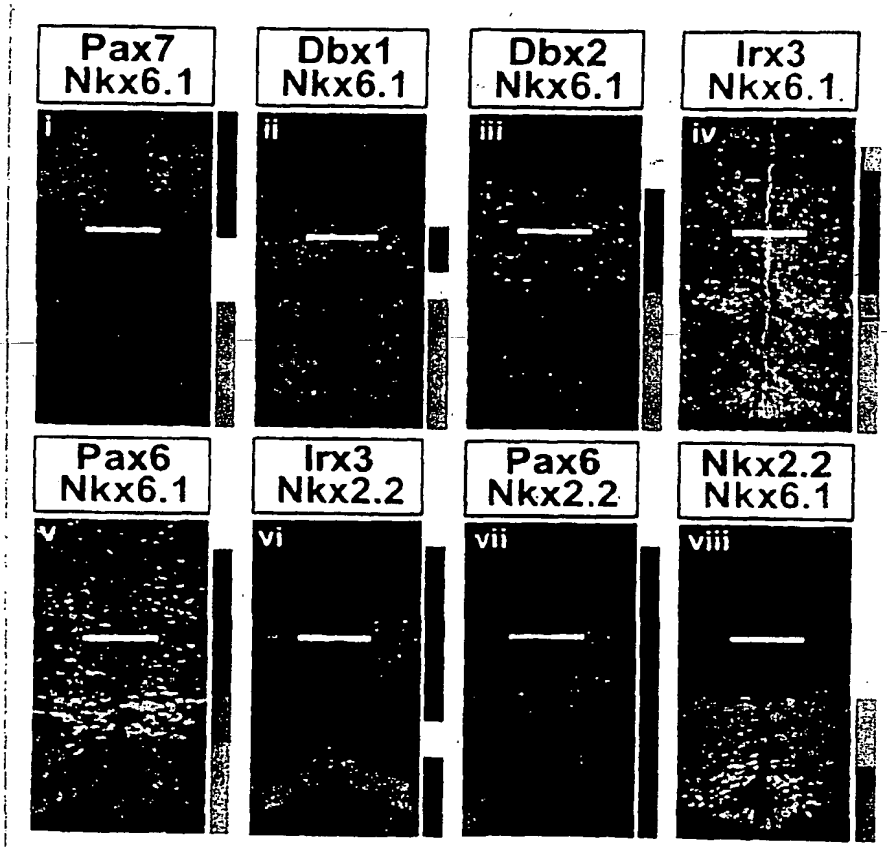

Pax7
Nkx6.1
i
Dbx1
Nkx6.1
ii
Dbx2
Nkx6.1
iii
Irx3
Nkx6.1
iv
Pax6
Nkx6.1
v
Irx3
Nkx2.2
vi
Pax6
Nkx2.2
vii
Nkx2.2
Nkx6.1
viii

FIGURE 2A

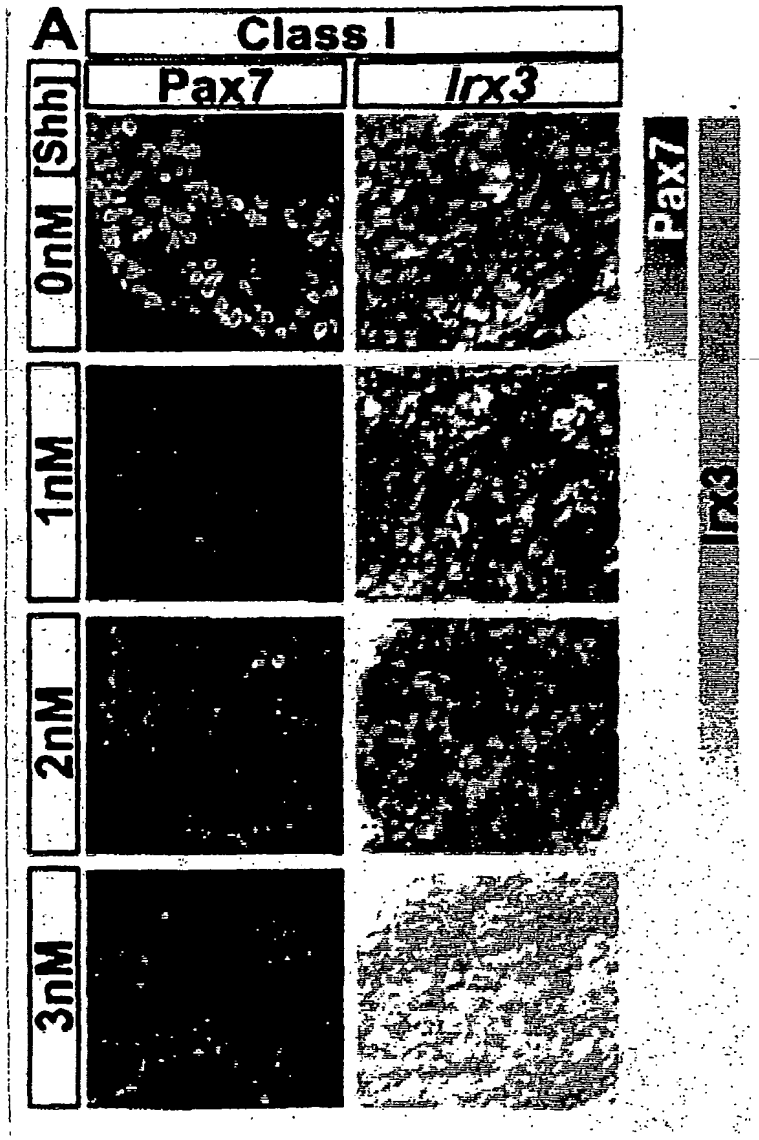


FIGURE 2B

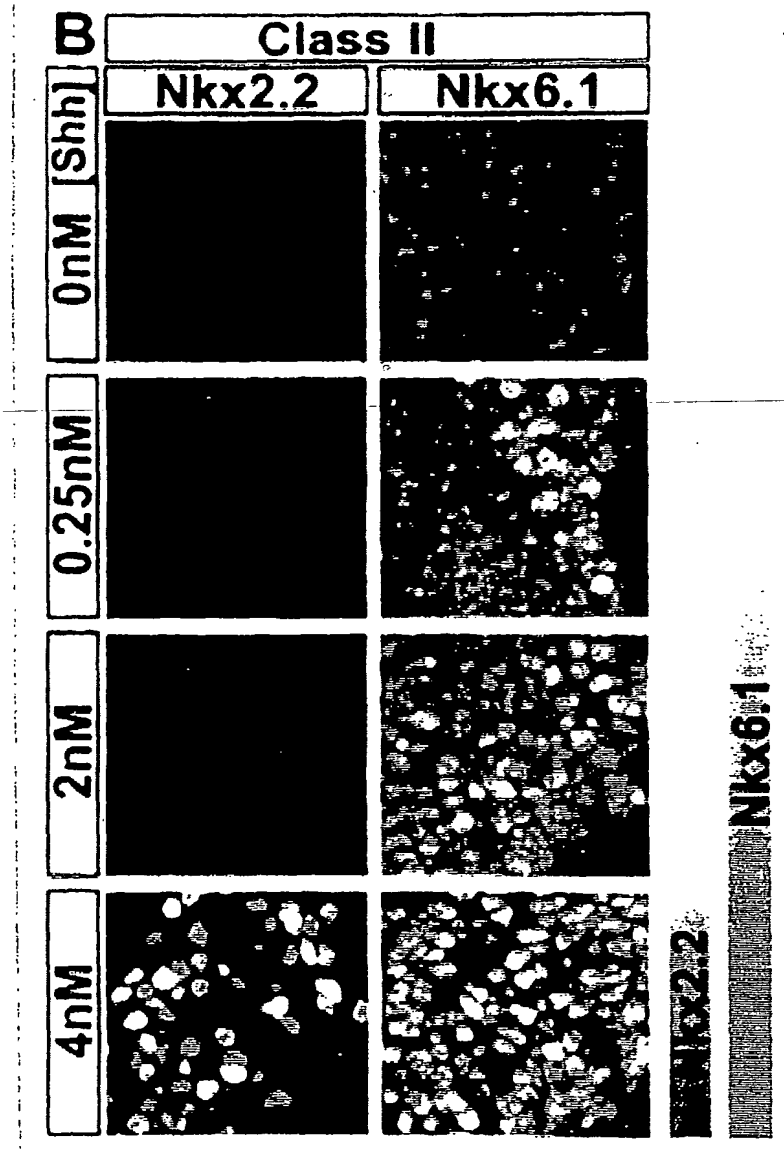
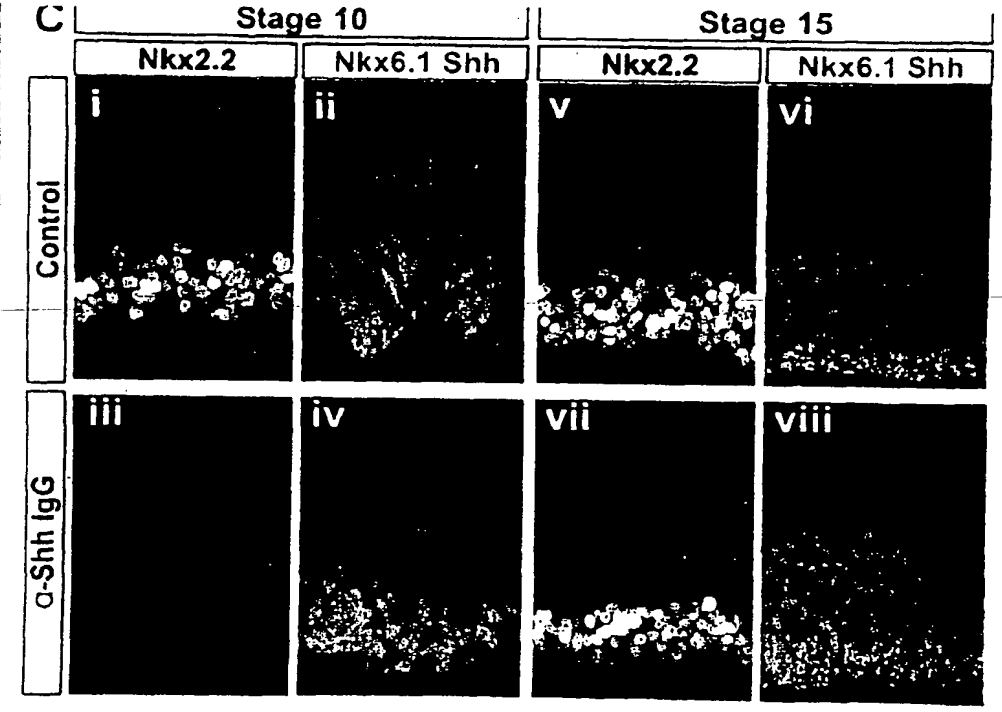


FIGURE 2C


C
Stage 10
Stage 15
Nkx2.2
Nkx6.1 Shh
Nkx2.2
Nkx6.1 Shh
Control
α-Shh IgG
i
ii
v
vi
iii
iv
vii
viii

6/30

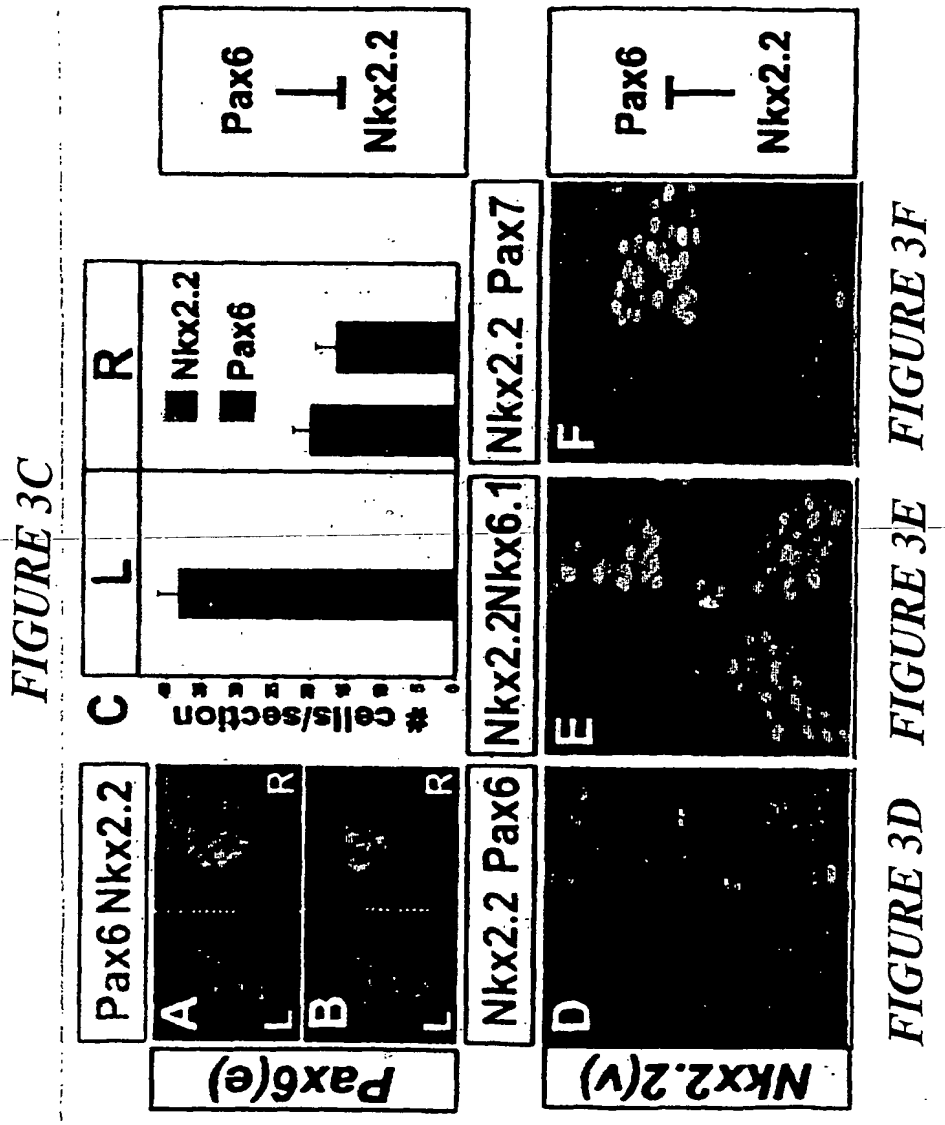


FIGURE 3A

FIGURE 3B

FIGURE 3C

FIGURE 3D

FIGURE 3E

FIGURE 3F

7/30

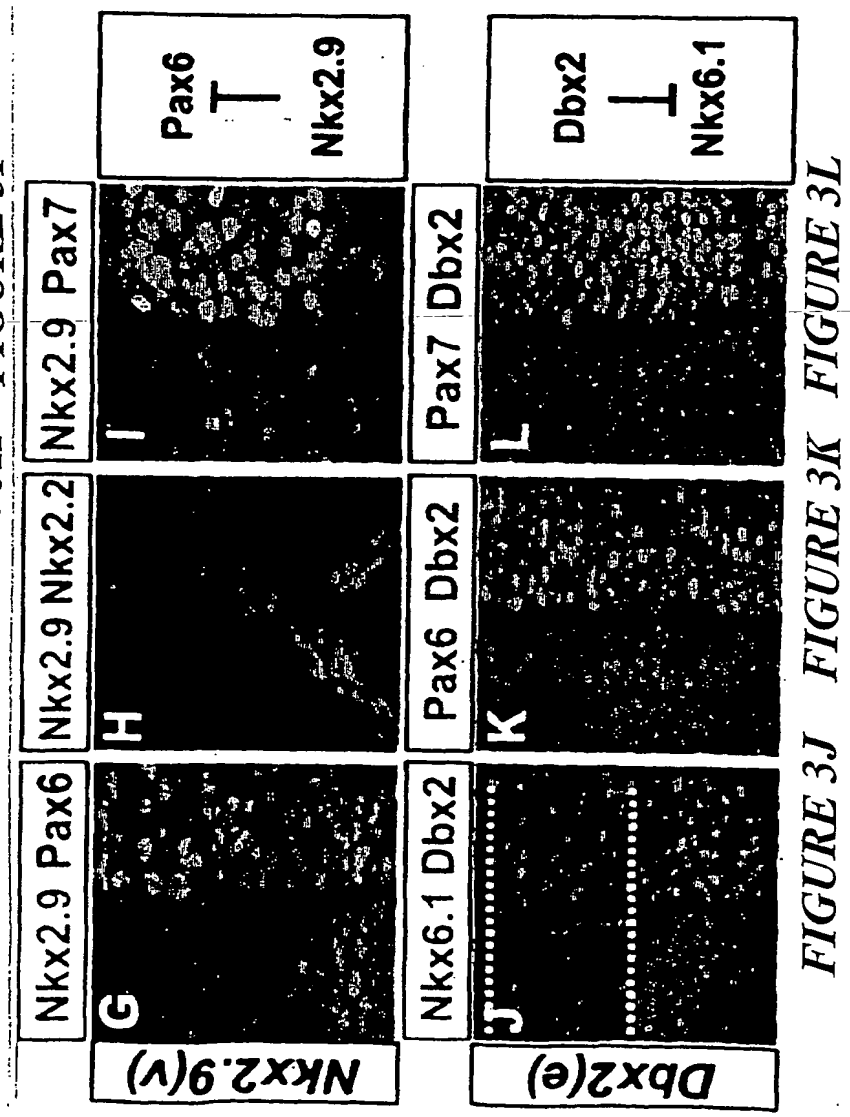


FIGURE 3G FIGURE 3H FIGURE 3I

FIGURE 3J FIGURE 3K FIGURE 3L

8/30

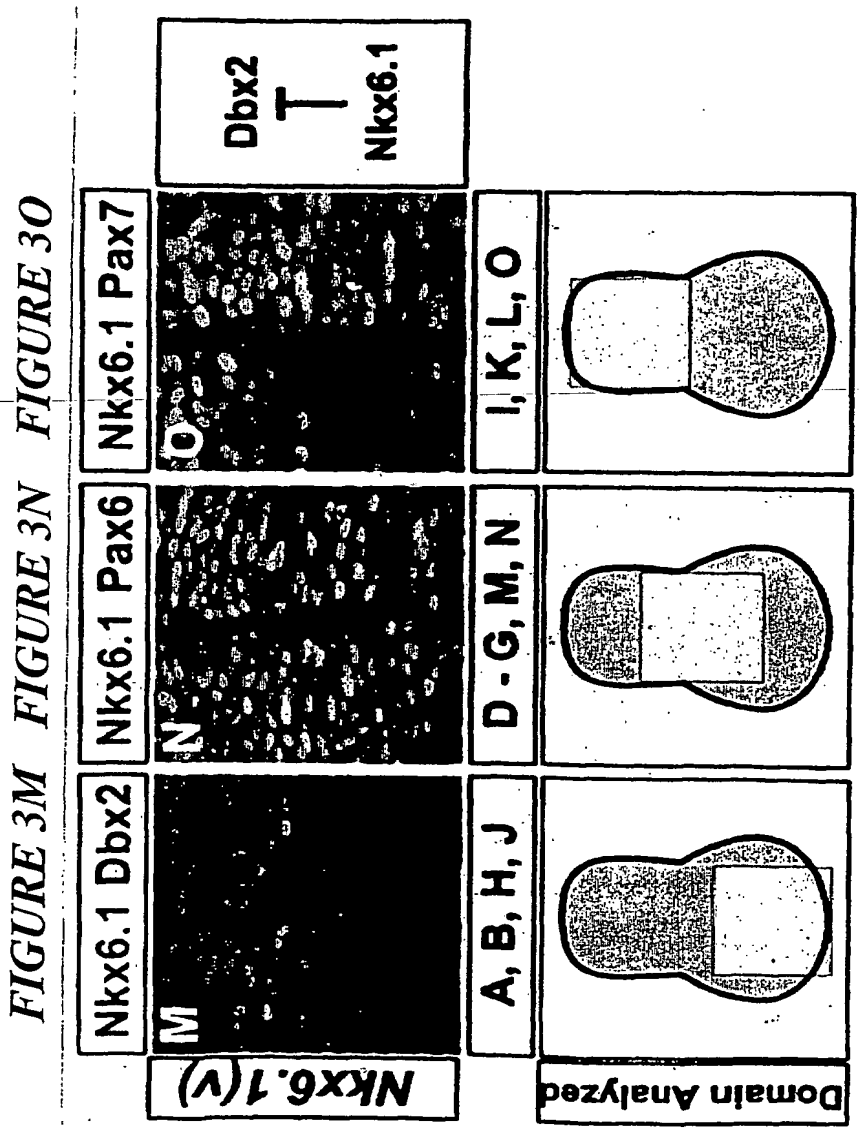


FIGURE 3M FIGURE 3N FIGURE 3O

9/30

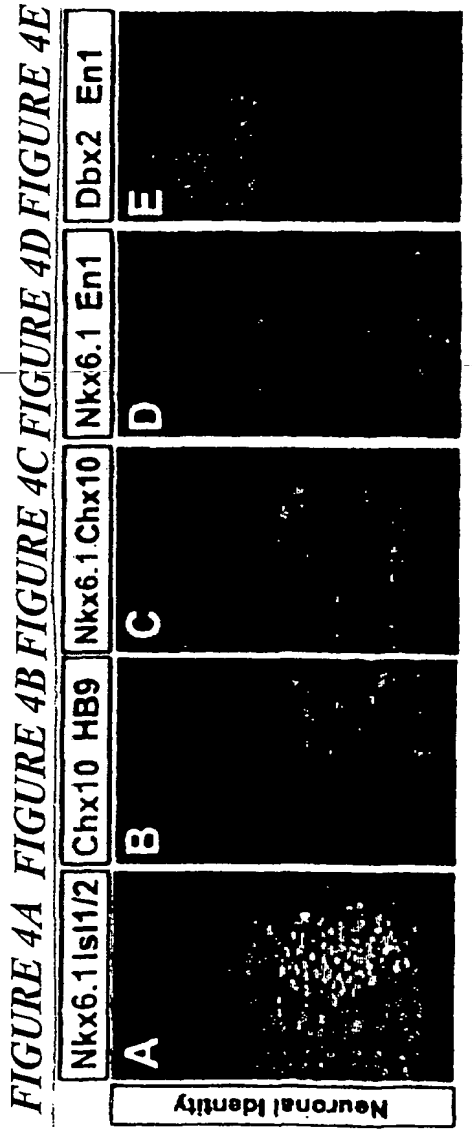


FIGURE 4A FIGURE 4B FIGURE 4C FIGURE 4D FIGURE 4E

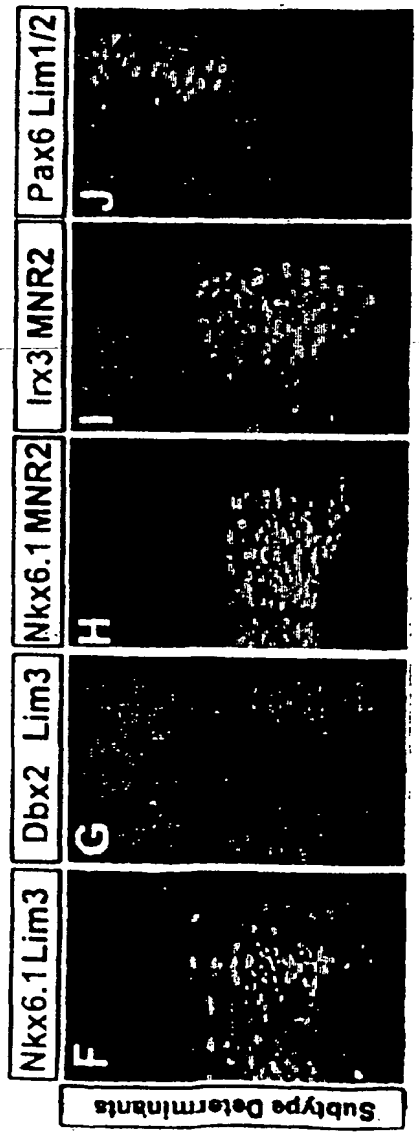


FIGURE 4F FIGURE 4G FIGURE 4H FIGURE 4I FIGURE 4J

10/30

FIGURE 4K

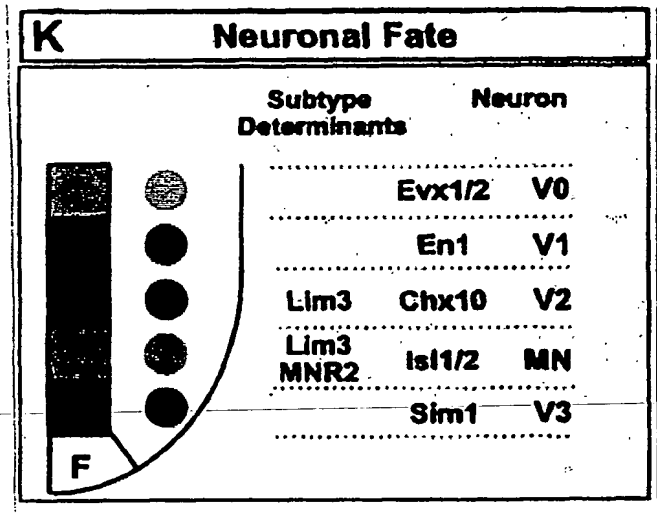


FIGURE 4L

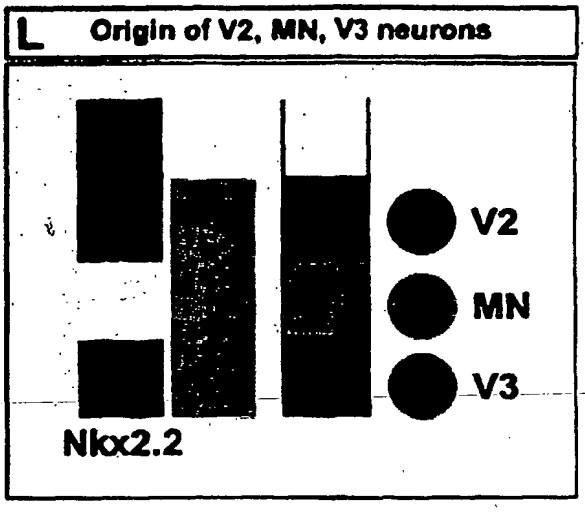


FIGURE 5A

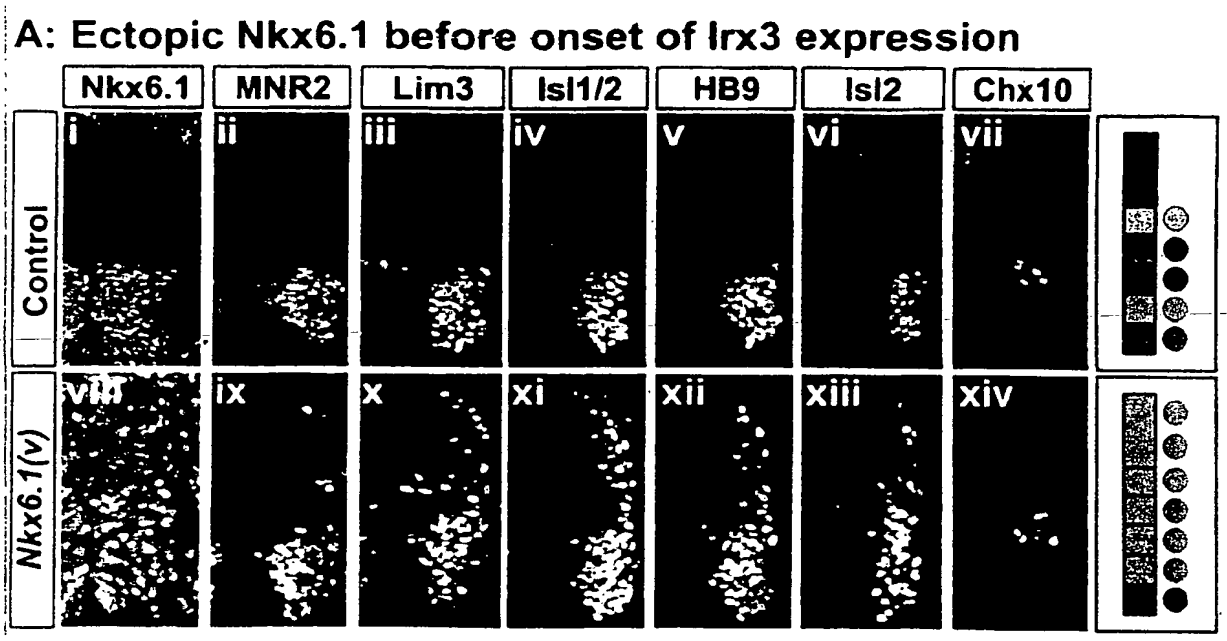
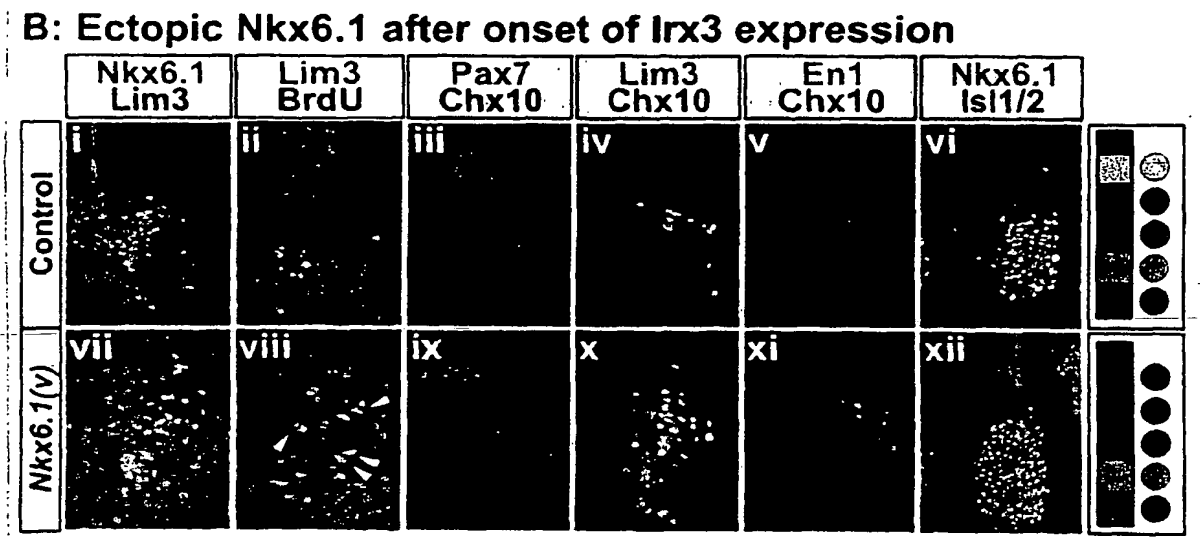
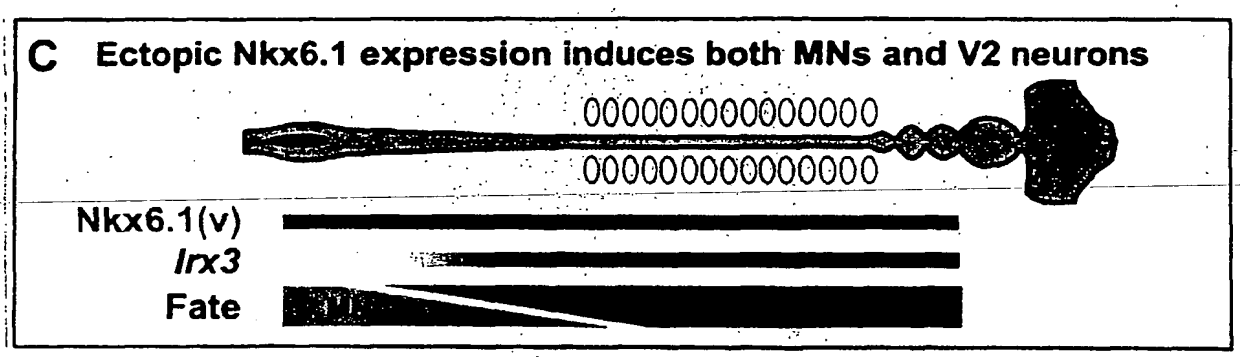

A: Ectopic Nkx6.1 before onset of Irx3 expression
Nkx6.1
MNR2
Lim3
Isl1/2
HB9
Isl2
Chx10
Control
Nkx6.1(v)
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii
xiii
xiv

FIGURE 5B


B: Ectopic Nkx6.1 after onset of Irx3 expression
Nkx6.1 Lim3
Lim3 BrdU
Pax7 Chx10
Lim3 Chx10
En1 Chx10
Nkx6.1 Isl1/2
Control
Nkx6.1(v)
i
ii
iii
iv
v
vi
vii
viii
ix
x
xi
xii

13/30

FIGURE 5C



14/30

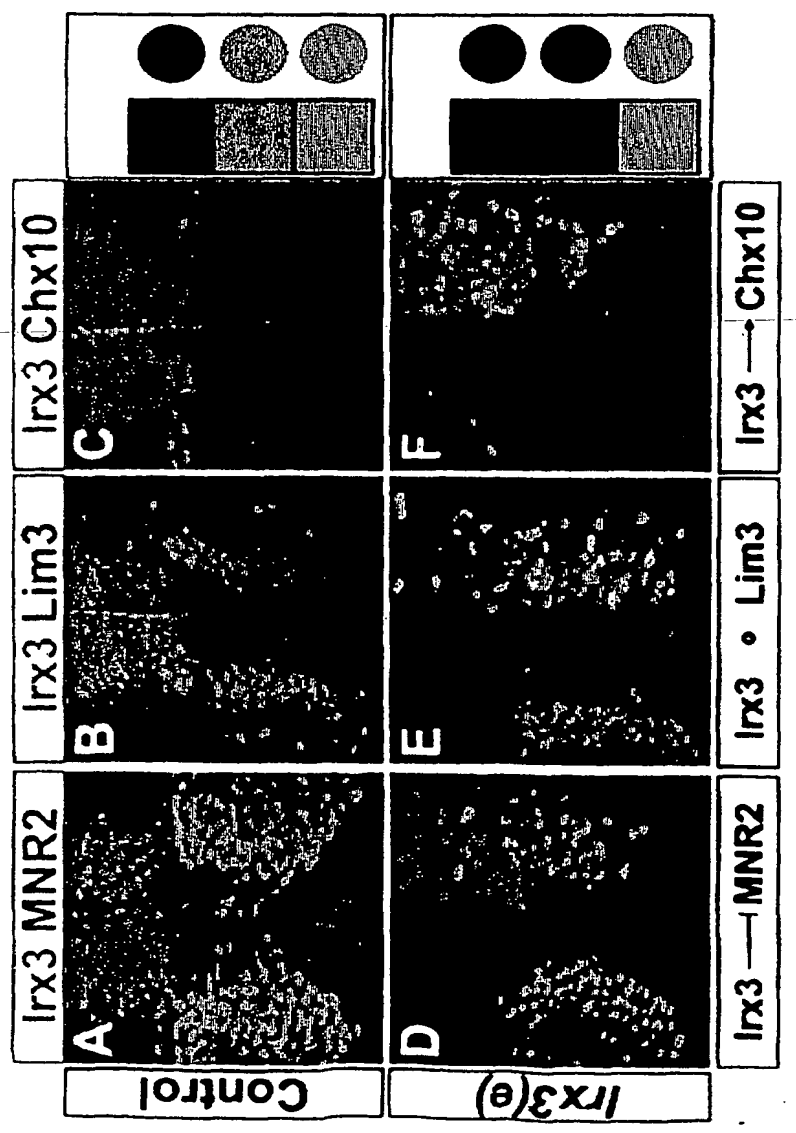
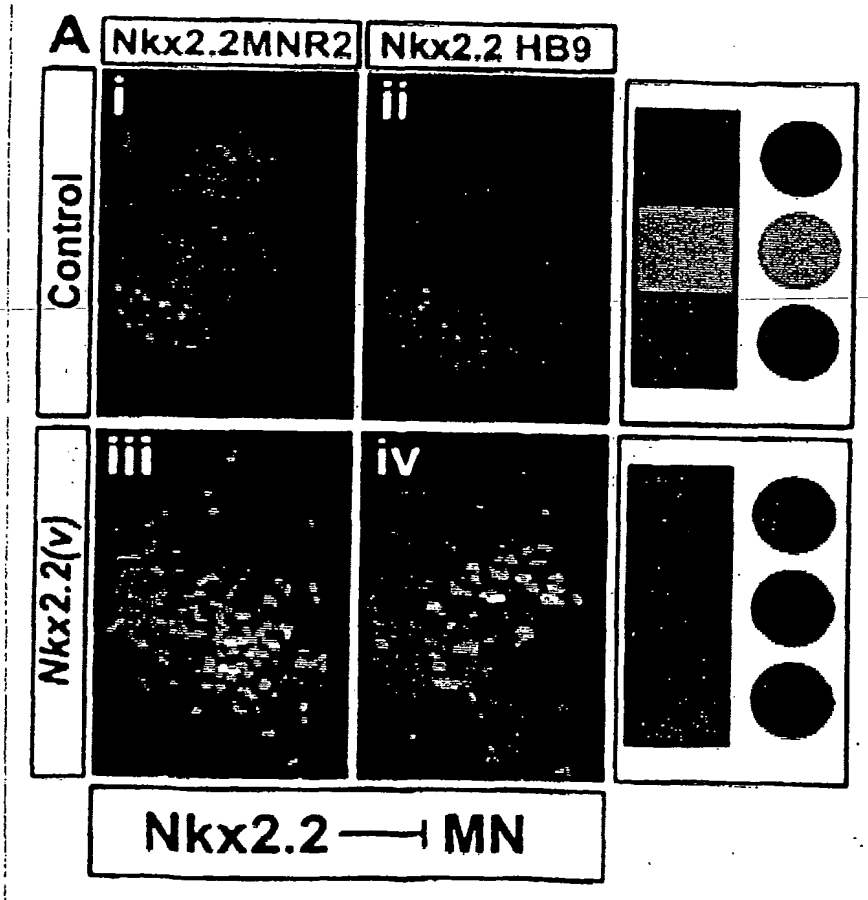


FIGURE 6A FIGURE 6B FIGURE 6C

FIGURE 6D FIGURE 6E FIGURE 6F

FIGURE 7A



16/30

FIGURE 7B

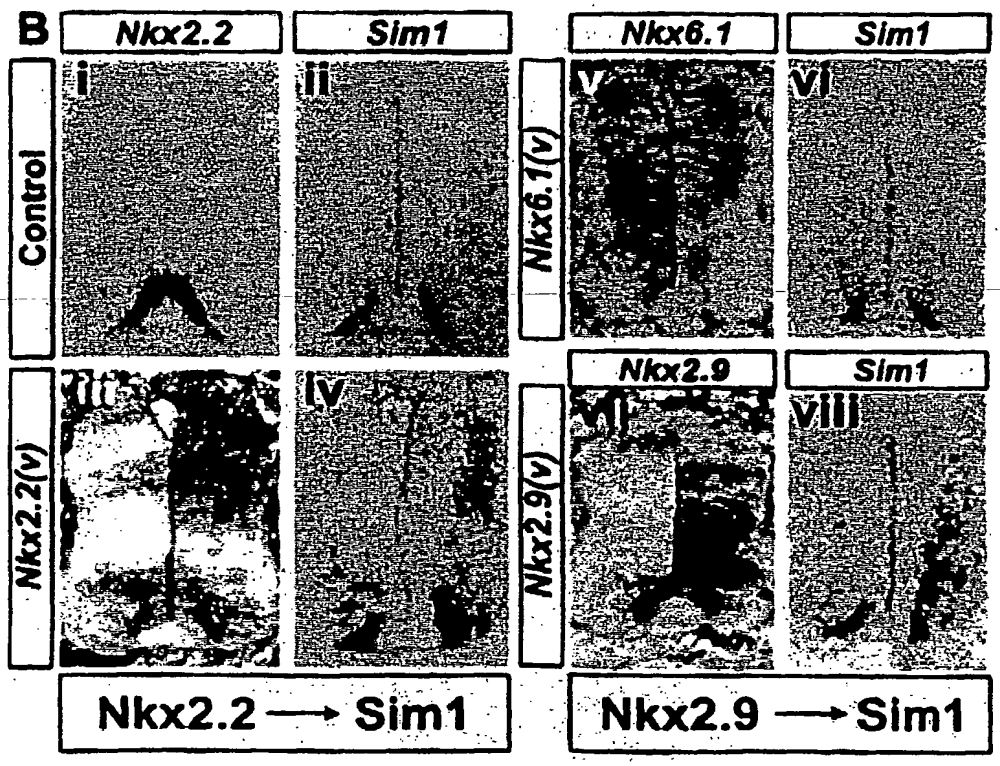


FIGURE 8A

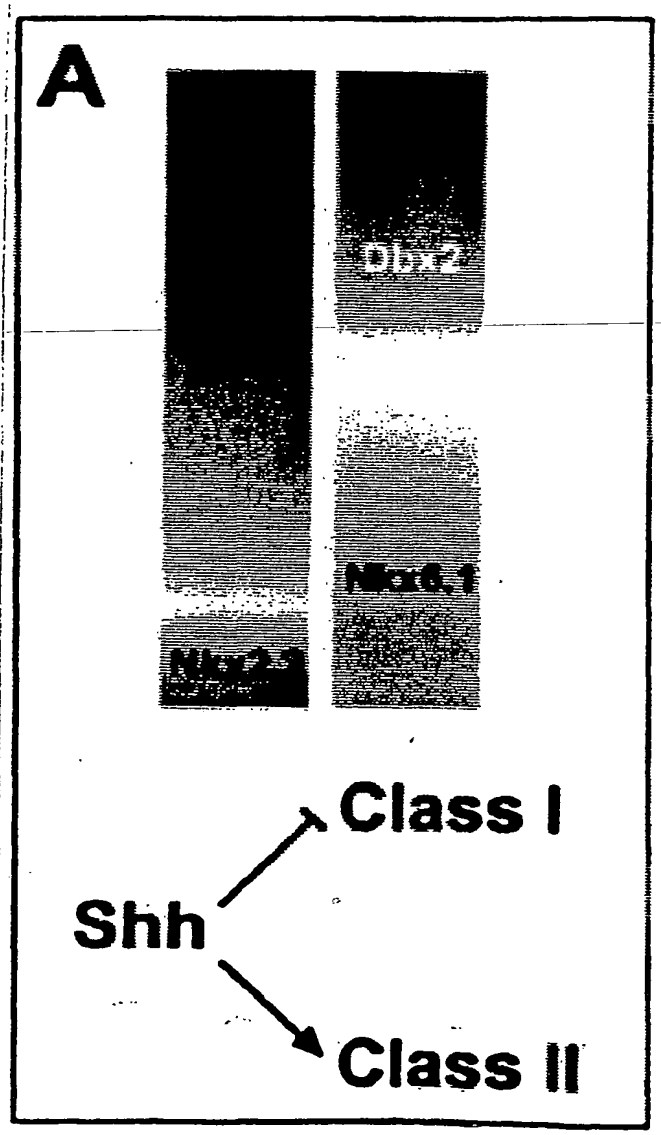


FIGURE 8B

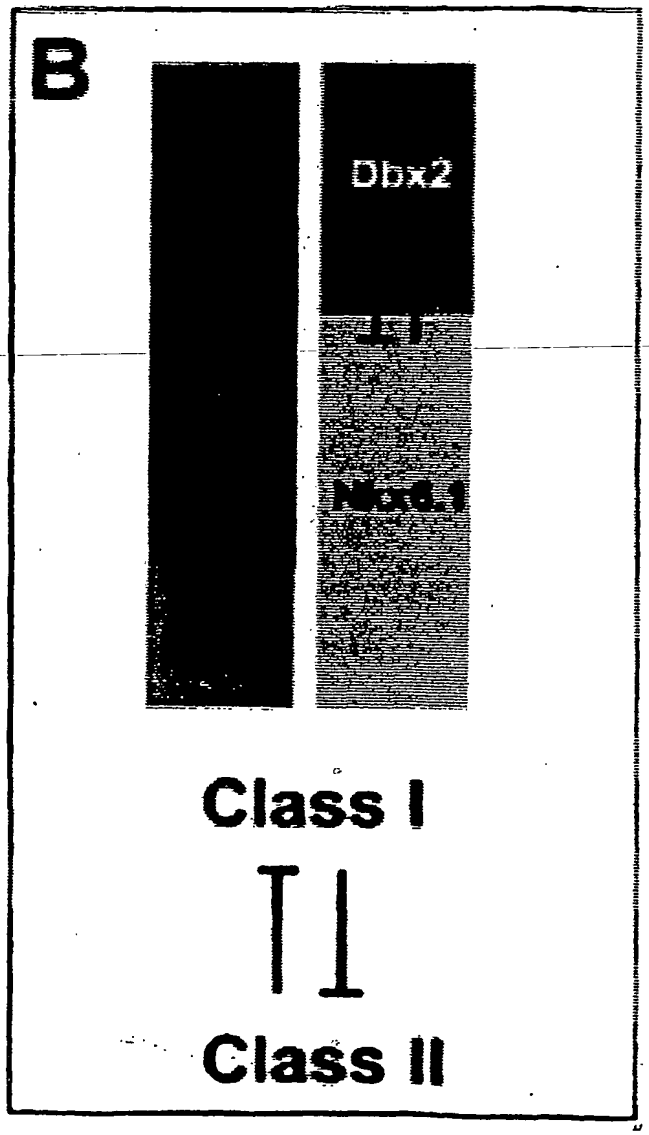
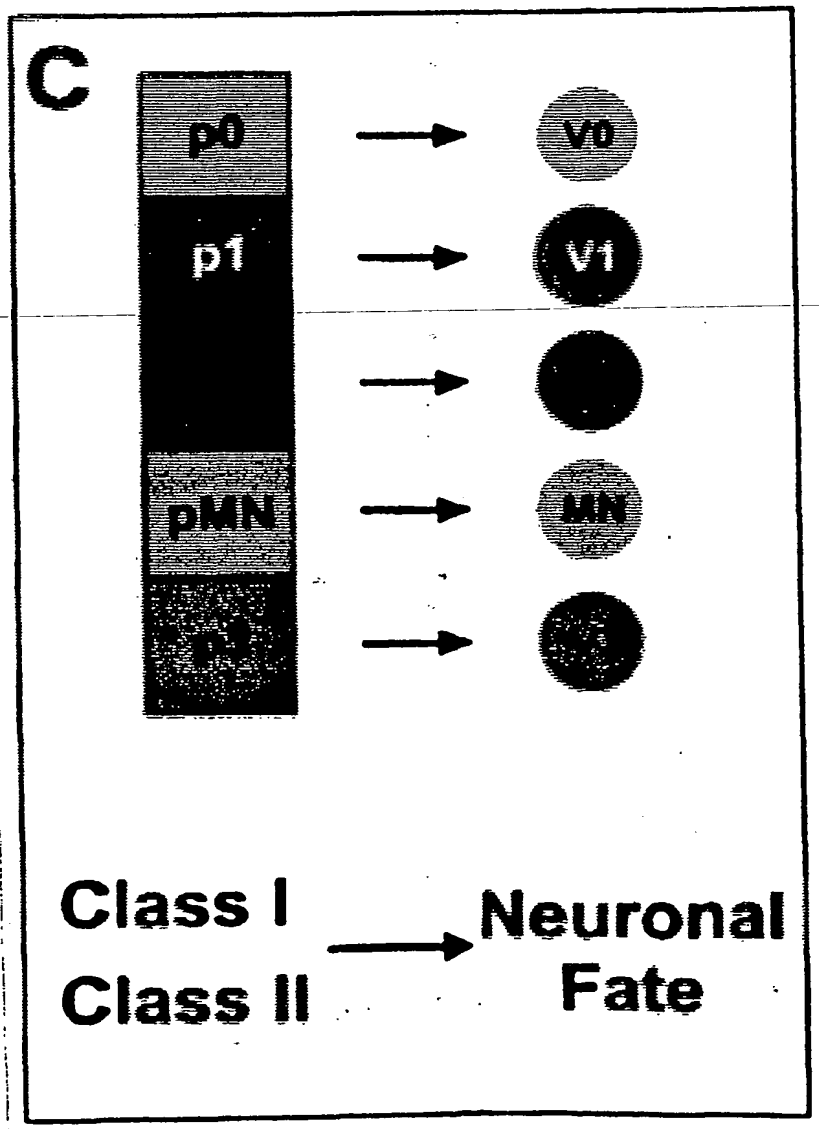


FIGURE 8C



20/30

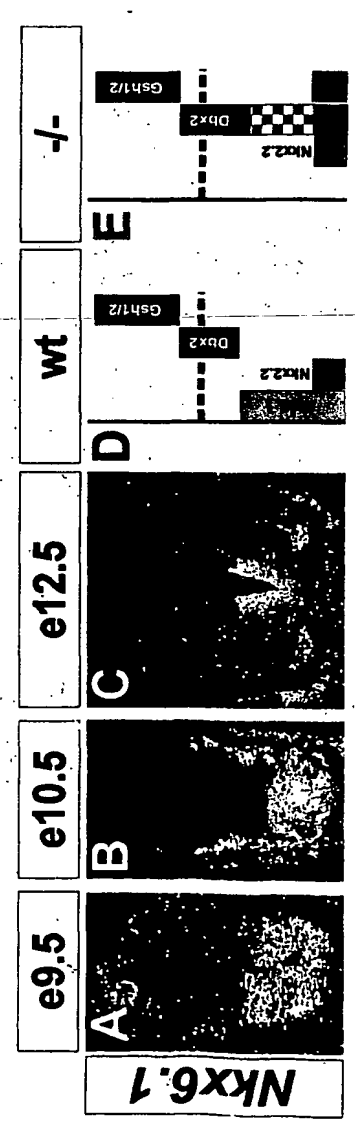


FIGURE 9A FIGURE 9B FIGURE 9C FIGURE 9D FIGURE 9E

21/30

FIGURE 9F *FIGURE 9G* *FIGURE 9H* *FIGURE 9I*

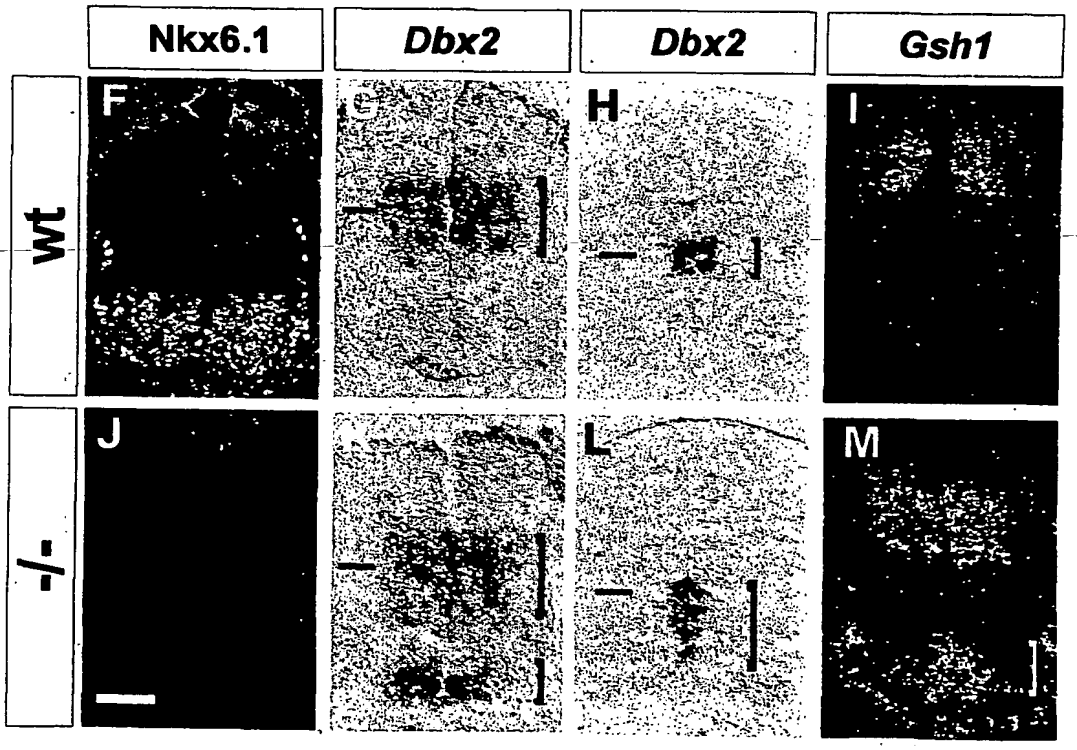


FIGURE 9J *FIGURE 9K* *FIGURE 9L* *FIGURE 9M*

22/30

FIGURE 9N FIGURE 9O FIGURE 9P FIGURE 9Q

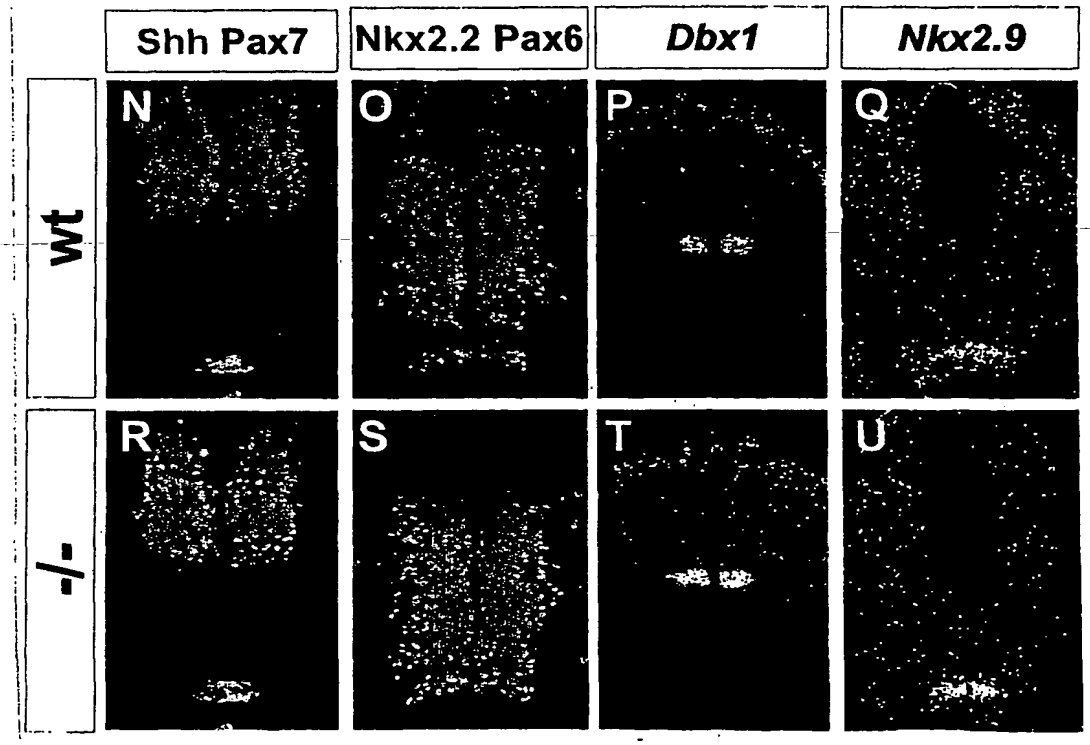


FIGURE 9R FIGURE 9S FIGURE 9T FIGURE 9U

FIGURE 10A *FIGURE 10B* *FIGURE 10C* *FIGURE 10D*

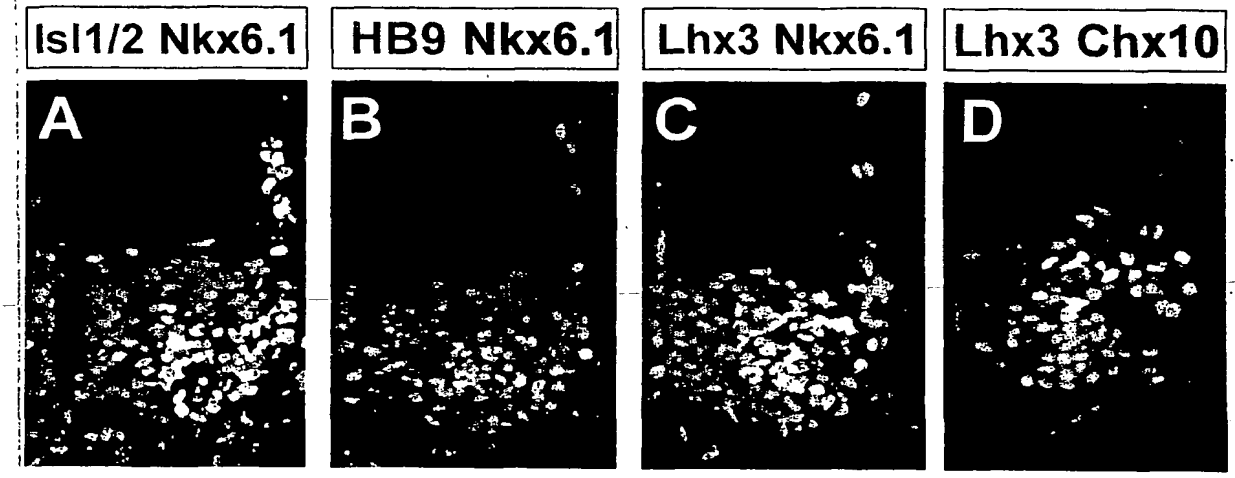


FIGURE 10E FIGURE 10F FIGURE 10G FIGURE 10H

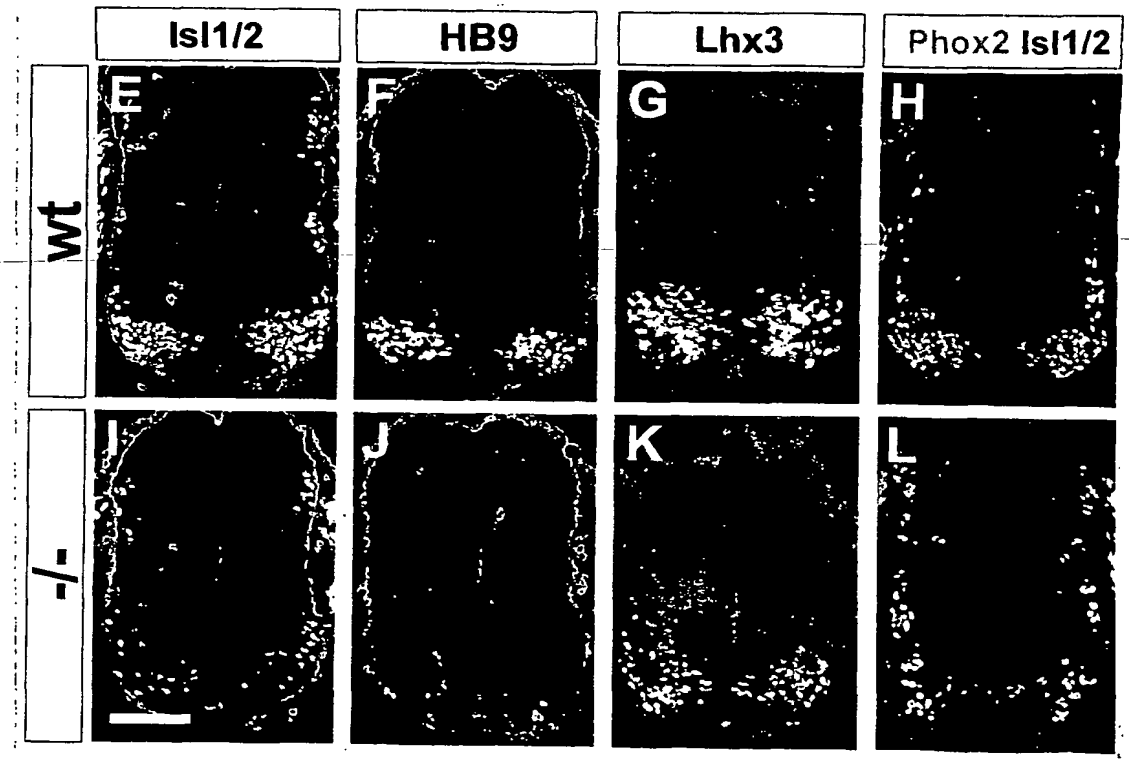


FIGURE 10I FIGURE 10J FIGURE 10K FIGURE 10L

25/30

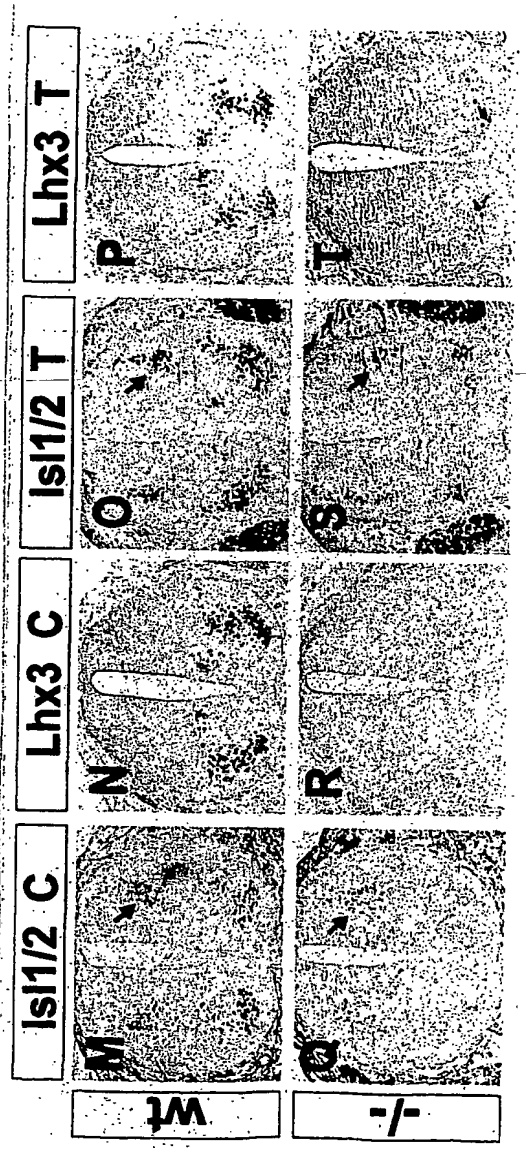


FIGURE 10M FIGURE 10N FIGURE 10O FIGURE 10P

FIGURE 10Q FIGURE 10R FIGURE 10S FIGURE 10T

26/30

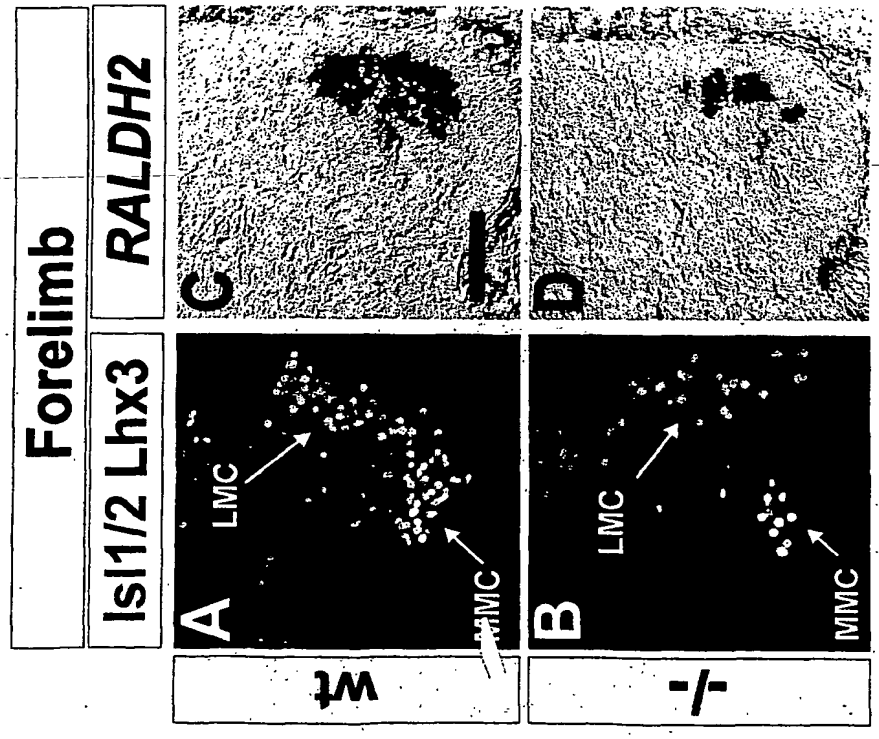


FIGURE 11A

FIGURE 11B

FIGURE 11C

FIGURE 11D

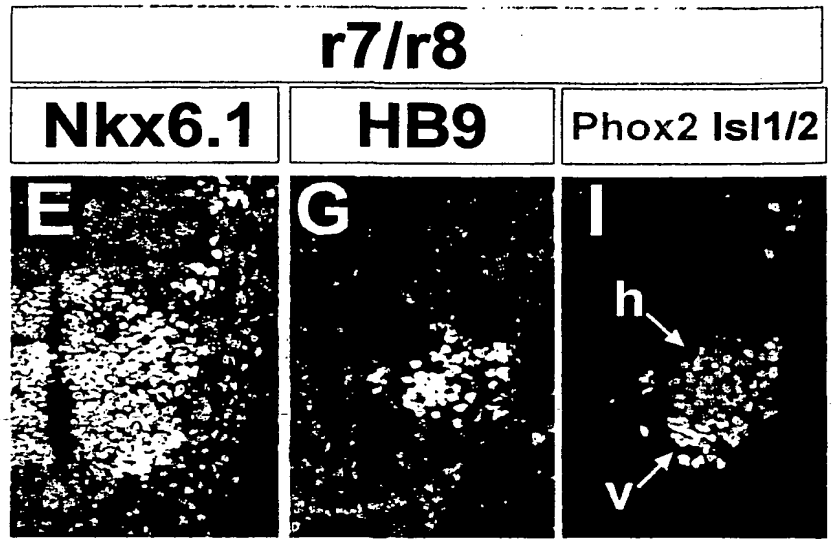
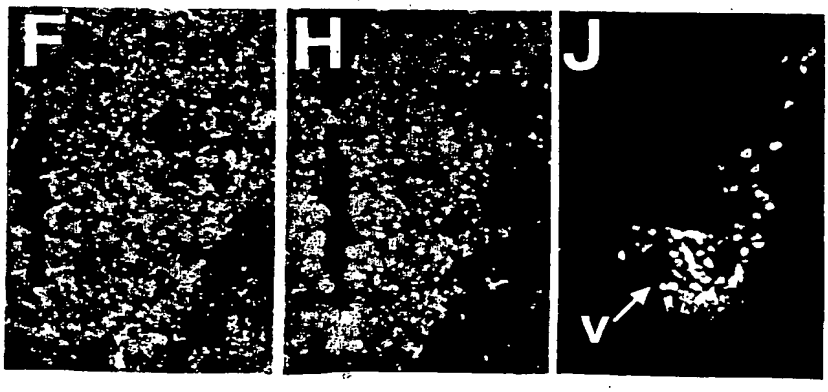


FIGURE 11E *FIGURE 11G* *FIGURE 11I*

FIGURE 11F *FIGURE 11H* *FIGURE 11J*



28/30

FIGURE 12A FIGURE 12C FIGURE 12E

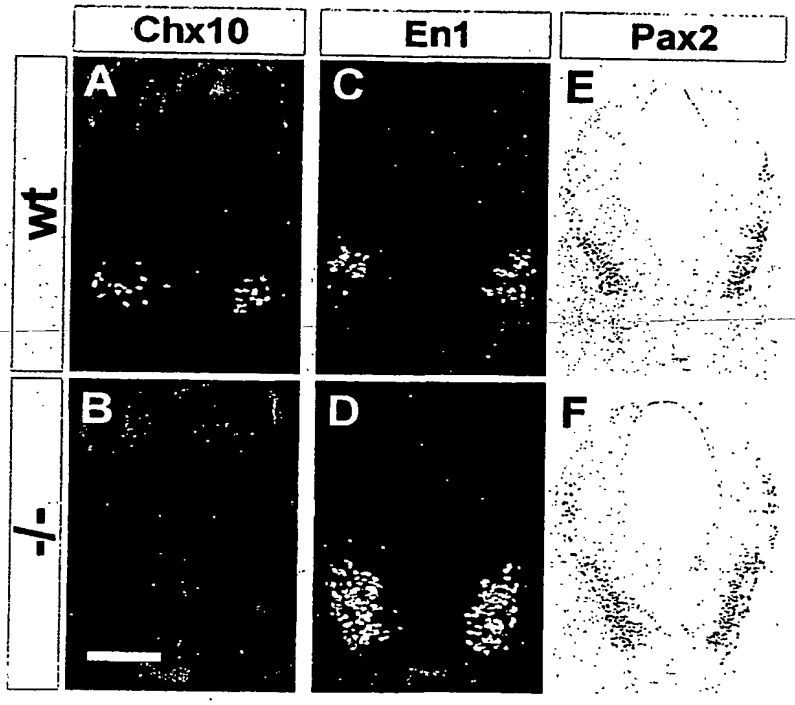


FIGURE 12B FIGURE 12D FIGURE 12F

FIGURE 12G FIGURE 12I FIGURE 12K

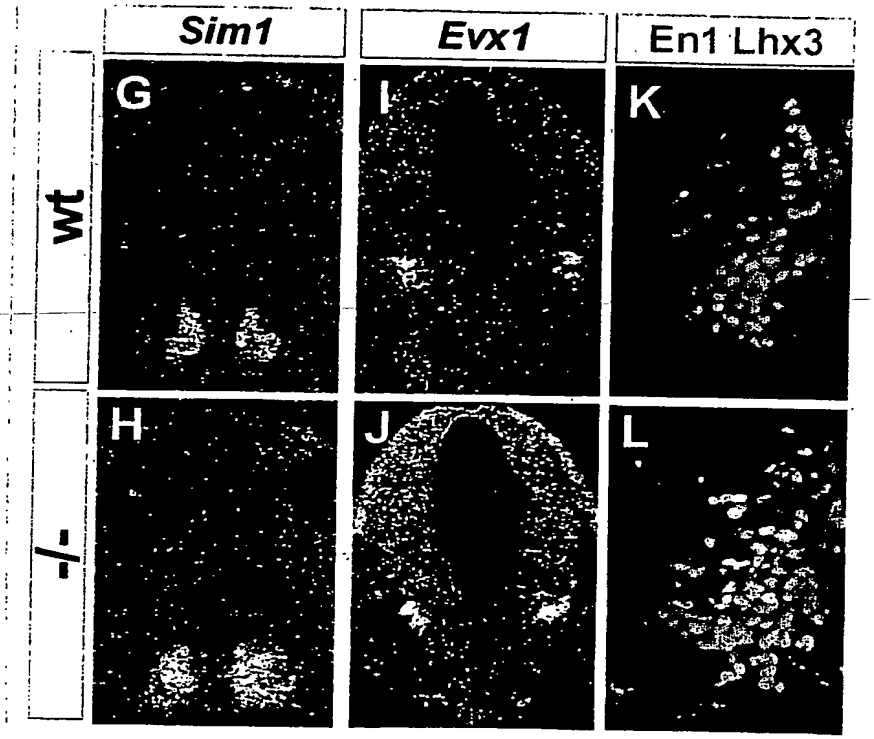


FIGURE 12H FIGURE 12J FIGURE 12L

30/30

FIGURE 13A

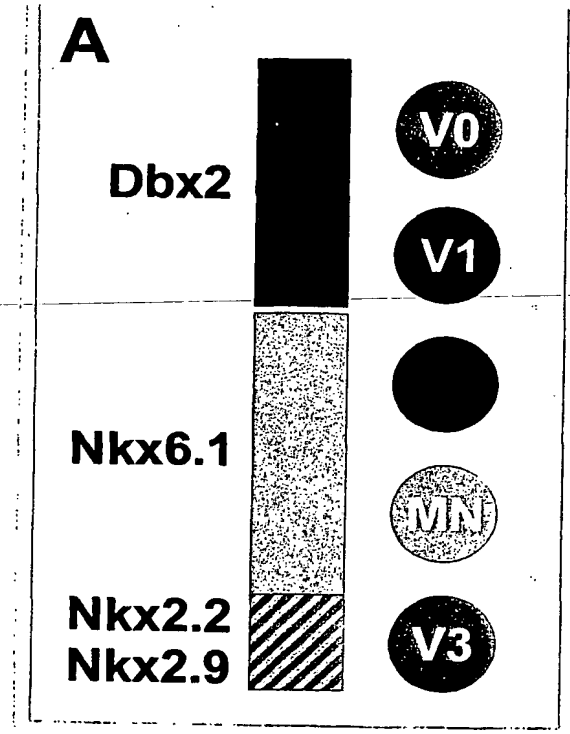
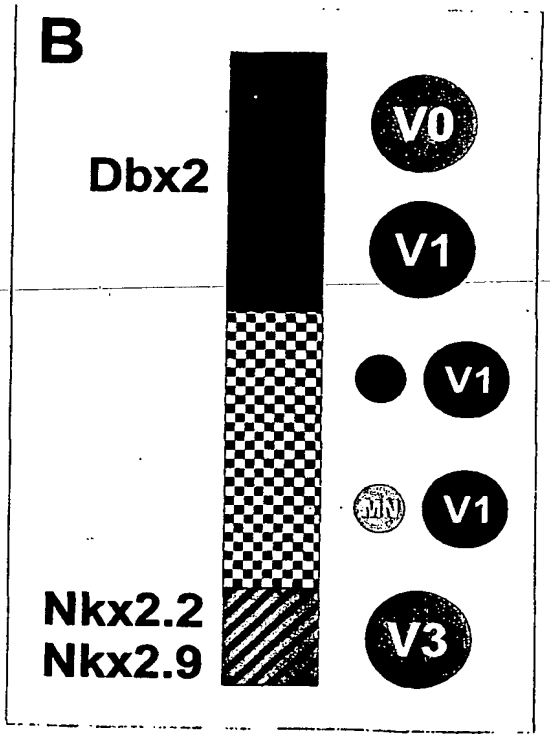


FIGURE 13B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/15290

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :Please See Extra Sheet.
US CL :424/93.1, 93.2, 93.21; 435/4, 325, 366, 368, 440, 455
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) .

U.S. : 424/93.1, 93.2, 93.21; 435/4, 325, 366, 368, 440, 455

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BRISCOE et al. Homeobox gene Nkx2.2 and specification of neuronal identity by graded Sonic hedgehog signalling. Nature. 15 April 1999, Vol. 398, pages 622-627, entire document.	1-31
A	LUMSDEN et al. Patterning the vertebrate neuraxis. Science. 15 November 1996, Vol. 274, pages 1109-1115, entire document.	1-31
A	QIU et al. Control of anteroposterior dorsoventral domains of Nkx-6.1 gene expression relative to other Nkx genes during vertebrate CNS development. Mechanisms of Development. 1998, Vol. 72, pages 77-88, entire document.	1-31

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier document published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search 27 JULY 2001	Date of mailing of the international search report 29 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer ANNE-MARIE BAKER, PH.D. Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/15290

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ROWITCH et al. Sonic hedgehog regulates proliferation and inhibits differentiation of CNS precursor cells. J. Neurosci. 15 October 1999, Vol. 19, No. 20, pages 8954-8965, entire document.	1-31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/15290

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (7):

A01N 63/00; A61K 48/00; C12N 5/00, 5/02, 5/08, 15/00, 15/63; C12Q 1/00

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

WEST
Dialog (file: medicine)
search terms: Nkx6.1, Nkx2.2, Irx3, homeodomain, transcription factor, neural, stem cell, Nkx2.9, neuron, degenerat?, retrovir?, V2, V3